IMMUNOLOGICAL STUDIES ON BOVINE BABESIOSIS WITH PARTICULAR REFERENCE TO BRAZIL, USING *IN VITRO* CULTURE-DERIVED ANTIGENS

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The study presented in this thesis is entirely the product of my own work except where specifically stated in the text and in the acknowledgements section.

Lygia M.F.Passos

To Sofia and Pedro

for reminding me that life has a most beautiful meaning.

CONTENTS

CHAPTER FOUR
IN VITRO CULTURE STUDIES

CHAPTER SIX
IMMUNOCHEMICAL CHARACTERISATION OF STOCKS OF *B.BOVIS* AND *B.BIGEMINA*

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

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The aim of the present study was to develop enzyme linked immunosorbent assays (ELISAs) for detection of specific antibodies against *Babesia bovis* and *B.bigemina* for use in epidemiological studies of bovine babesiosis in Brazil. These tests were developed using purified proteins of each parasite, which were identified as species-specific in a comprehensive immunochemical characterisation of different stocks of *Babesia* parasites.

The review of the literature on bovine babesiosis covers the geographical distribution, transmission and life cycle, and *in vitro* cultivation of both species, as well as immunology, diagnosis, epidemiology and control of the disease and its current situation in Brazil.

The thesis first describes a series of studies on the *in vitro* culture of *B.bovis*, which include the evaluation of the effect of different sera on *in vitro* growth, the incorporation of feeder cells (bovine aortic endothelial and mouse peritoneal cells) into cultures, cloning of one isolate by *in vitro* limiting dilutions, initiation of cultures from low parasitaemia blood, and several attempts to establish African isolates of *B.bigemina in vitro*.

Methods for concentration of *Babesia* infected red blood cells and free parasites were then examined. These included the use of differential hypotonic lysis, density gradient centrifugation, induction of free merozoites into culture supernatant and techniques for fractionation of exoantigens present in culture supernatant, namely high performance liquid chromatography.

Somatic components and exoantigens of different stocks of parasites were characterised immunochemically by Western immuno-blotting, and immunoprecipitation of ^{35}S-methionine labelled proteins by acrylamide gel electrophoresis, using a wide variety of sera which included calf sera experimentally raised against different stocks of each parasite, sera collected in the field in Brazil and a panel of monoclonal antibodies previously produced against *B.bovis* and characterised by IFAT, Western immuno-blotting and ELISA in the present study. The aim of the immunochemical characterisation was to identify antigens common to all stocks yet species-specific with potential use for the development of ELISAs. Antigenic diversity amongst stocks of *B.bovis* is reported.

For the development of the ELISAs, calf serum samples of known status and serum samples collected in endemic areas in Brazil, Malawi and Mozambique were utilised to compare assays using crude antigen preparations of each *Babesia* species and purified specific proteins. Four *B.bovis* stock-conserved proteins from somatic components were electro-eluted from acrylamide gels and used to develop an ELISA. Amongst these, a 56 kDa protein was found to be a potential candidate to replace the crude parasite extract in immunodiagnostic assays.

Several *B.bigemina* specific proteins were also identified in the immunochemical analysis and four candidates were electro-eluted from acrylamide gels. However all purified *B.bigemina* proteins cross-reacted with *B.bovis* antisera in ELISA. The complementary use of a crude and cross-reactive *B.bigemina* antigen together with the 56 kDa *B.bovis*-specific protein in ELISA to elucidate the epidemiology of these diseases is recommended.

Future prospects and potential application in Brazil of these ELISA antigens for use in the detection of anti-*B.bovis* and anti-*B.bigemina* antibodies are discussed with particular reference to their role in defining the epidemiology of bovine babesiosis in the dairy cattle of Minas Gerais State, Brazil.

ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
BAE	Bovine aortic endothelium
BPA	Bovine plasma albumin
CF	Complement fixation
c.p.m.	Counts per minute
CTVM	Centre for Tropical Veterinary Medicine
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAO/IAEA	Food and Agriculture Organisation of the United Nations/International Atomic Energy Agency
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
g	Gravitational force
GPI	Glucose phosphate isomerase
HBSS	Hank's balanced salt solution
HPLC	High performance liquid chromatography
IFAT	Indirect fluorescent antibody test
IHA	Indirect heamagglutination assay
ILRAD	International Laboratory for Research on Animal Diseases
iRBC	Infected red blood cells
kDa	Kilodalton
MASP	Microaerophilous stationary phase
McAb	Monoclonal antibody
MPWC	Mouse peritoneal wash cells
NBS	Normal bovine serum
NK	Natural killer
NP-40	Nonidet P-40
OD	Optical density
p	Probability
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	PBS-Tween
PCV	Packed cell volume
p.i.	Post-infection

PMSF	Phenylmethyl-sulfonyl fluoride
pp	Precipitate
PVP	Polyvinylpyrolidone
RBC	Red blood cells
RIA	Radioimmunoassay
rpm	Revolutions per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SELISA	Slide ELISA
SIP	Stock isotonic Percoll
TBS	Tris buffered saline
TCA	Trichloroacetic acid
TEMED	N N N'N-tetramethylethylenediamine
Th	T helper
TLCK	Tosyl-lysine chloromethyl ketone
TRIS	Hydroxymethyl amino methane
WBC	White blood cell

LIST OF TABLES

LIST OF FIGURES

CHAPTER SIX

CHAPTER SEVEN

CHAPTER EIGHT

CHAPTER ONE

INTRODUCTION

microplus which transmits *B.bovis* and *B.bigemina*. Brazil is located in an area with climatic conditions favourable for the development of *B.microplus* and therefore its entire bovine population (around 140 million animals) is potentially at risk from these tick-borne diseases. Anaplasmosis, a disease of similar pathogenesis and pathogenicity caused by the rickettsial organism *Anaplasma marginale*, is the third disease of a triad transmitted by this tick causing a disease syndrome known in South America as 'tristeza parasitaria' (Fonseca & Braga, 1923).

As early as 1923 this syndrome was reported by Fonseca and Braga as being one of the major constraints to the development of the Brazilian cattle industry, affecting calves and genetically improved animals, which it was necessary to immunise before introducing them into tick-infested areas (Fonseca & Braga, 1923). These two Brazilian veterinarians made the first substantial contribution to the understanding of several aspects of these diseases including their pathogenesis, clinical symptoms, treatment and immunisation in Brazil. They also stressed the need for adoption of measures to control the tick vector in the face of increased importations of *Bos taurus* cattle with the aim of upgrading local herds. Nowadays, surveys carried out amongst dairy farmers in Brazil still cite tick-borne diseases as second only in importance to diarrhoea among diseases affecting calves. Although little information on economic losses due to each individual tick-borne disease is available, it is clear to veterinary practitioners that babesiosis makes a substantial contribution to the reduction in the farmer's income.

Due to the large extent of the Brazilian territory, a variety of topographic and climatic conditions occur; consequently, diverse epidemiological situations exist which require different control measures. As little information concerning the epidemiology of bovine babesiosis throughout Brazil is available, it becomes difficult to propose control measures to suit best each particular epidemiological situation. In order to propose appropriate control measures, it is essential to understand the various factors involved in the epidemiology of bovine babesiosis. Serological surveys are commonly used to provide relevant information regarding infection prevalence which may make substantial contributions to decisions on strategies to control infections in a particular area.

Despite the fact that a large number of serological tests can be used as the basis for epidemiological surveys, there are some limitations, common to all immunodiagnostic tests based on the detection of antibodies, that may lead to misleading results and consequently recommendation of inappropriate control measures. One example of this is given by the difficulty in discriminating between antibodies generated against each species of *Babesia*, since there is usually cross-reactivity between *B.bovis* and *B.bigemina* in serological tests where crude parasite preparations are used. Prediction of prevalence and incidence of disease

based on results from serological tests may therefore not always be accurate. The reliability of serological tests increases as the specificity and sensitivity increase. Discrimination between the two infections in a particular area may assume great importance in cases where, for instance, immunisation programmes are to be implemented. If live vaccines are going to be used, it is important to define which *Babesia* species is needed for inclusion in the vaccine, particularly considering that different techniques are used for attenuation of each *Babesia* species. Thus, inclusion of one species unnecessarily in a vaccination programme may represent a substantial increase in cost of such activities for the individual farmers.

Therefore there is still a need for improvement of the diagnostic tests already developed and, as most of the cross-reactivity between related parasites is due to low specificity of antigens used for detection of antibodies, improvements in the specificity of antigens seems to be a sensible approach to achieve more reliable serological tests.

Thus, in the present study the main objective was to develop sensitive immunodiagnostic tests for indirect detection of *B.bovis* and *B.bigemina* infections for epidemiological surveys in endemic areas, using specific components of each parasite as antigens to replace crude parasite preparations in enzyme-linked immunosorbent assay (ELISA) systems. Such tests would be useful for a comprehensive study on the epidemiology of bovine babesiosis in areas of Brazil, such as Minas Gerais State, where the disease is a problem faced in the development of the cattle industry, currently with approximately 20 million animals.

The approach used to achieve this main aim was based on the identification of species-specific parasite components through detailed immunochemical characterisation of different stocks of each *Babesia* species, which would allow the identification and selection of relevant antigenic components for use in the development of antibody detection ELISAs for each parasite. The development of the tests was carried out in comparison with crude antigen preparations and the assays were validated by testing with panels of experimentally produced calf sera of known status and serum samples collected from cattle raised in areas endemic for bovine babesiosis.

Preceding the immunochemical characterisation of *Babesia* stocks, it was necessary to ensure a reliable source of both parasites. To avoid unnecessary use of experimental animals and to obtain reliable and reproducible parasite material at the Centre for Tropical Veterinary Medicine (CTVM), the *in vitro* culture systems for both *B.bovis* (Levy & Ristic, 1980) and *B.bigemina* (Vega et al, 1985a) were seen to be the most appropriate means of providing infected red blood cells (RBC) throughout the study. Thus a series of experiments was designed, at the beginning of the present investigation, in which the aim was to improve the *in vitro* cultivation system of *B.bovis* and to establish, *in vitro*, isolates of *B.bigemina* which had not been established as continuous cultures in the past.

Building on successful *in vitro* cultivation, the second stage of the study was directed towards the enrichment of *Babesia* from culture suspensions, providing parasite-rich preparations for the immunochemical analysis, in which several techniques were used for the identification of relevant species-specific parasite components (both somatic and exoantigens) with potential for use as immuno-diagnostic targets.

ELISA tests for detection of specific anti-*B.bovis* anti-*B.bigemina* antibodies were then developed using purified antigens and crude antigenic preparations of each parasite, and serum samples of known status and field samples collected in *Babesia* endemic areas .

CHAPTER TWO

LITERATURE REVIEW

2.1 THE PARASITES

2.1.1 Classification

The babesias are intraerythrocytic protozoan parasites of mammals. The classification of babesias has been a subject of debate for years. The Society of Protozoologists (Levine et al, 1980) proposed the following classification for these parasites as:

Phylum: Apicomplexa
Class: Sporozoa
Subclass: Piroplasmia
Order: Piroplasmida
Family: Babesiidae
Genus: *Babesia*

However, as the classification of the parasitic protozoa became unsatisfactory (Lee et al, 1985), a review was carried out resulting in a new classification proposed by Sleigh (1989). The original class of sporozoans contained a heterogeneous collection of spore-forming unicellular organisms, some of which are now classified in distinct phyla, leaving the remaining sporozoans in a single category regarded as Phylum Sporozoa containing three classes: Gregarina, Coccidea and Piroplasmea. The classification of the babesias in the order Piroplasmida, family Babesiidae and genus *Babesia* proposed by Levine remained the same. The systematics of parasitic protozoa comparing the classifications according to Levine (1980) and Sleigh (1989) has recently been reviewed by Cox (1991). From this, *Babesia*, according to Sleigh, would be classified as follows:

Phylum: Sporozoa
Class: Piroplasmea
Order: Piroplasmida
Family: Babesiidae
Genus: *Babesia*

2.1.2 History and geographical distribution

The babesias were first described by Babes in 1888 as *Haematococcus bovis* in cattle in Rumania (Babes, 1888) and were renamed *Babesia bovis* by Starcovici five years latter (Starcovici, 1893). In 1893 Smith and Kilborne proposed the name *Pyrosoma bigeminum* for the parasite causing Texas fever in the southern United States and first reported the transmission of a pathogenic protozoan by an invertebrate arthropod, the tick *Boophilus annulatus* (Smith & Kilborne, 1893). In 1934 Rees reported that both *B.bovis* and *B.bigemina* were involved with Texas fever (Rees, 1934).

In Argentina two strains of *Babesia* were identified by Lignieres in blood smears from cattle as causative agents of "tristeza"; one was large and similar to the organism described by Smith and Kilborne, while the other was smaller and more easily seen in capillaries of the kidneys and the meninges of the central nervous system (Lignieres, 1903). The small organism was named *Piroplasma argentina* (Lignieres, 1910), later known as *Babesia argentina*, until a consensus of parasitologists established that *B.bovis* and *B.argentina* were the same parasite and that the unique designation of *Babesia bovis* should be used thereafter (Brocklesby, 1978).

In 1911, M'Fadyean and Stockman reported the presence in England of a small intraerythrocytic parasite that they named *Piroplasma divergens* (later *Babesia divergens*) (M'Fadyean & Stockman, 1911), which was considered to be the same as *B.bovis* for some years, due to morphological similarities with that parasite. Another species of *Babesia*, which was morphologically very similar to *B.bigemina*, was first described as *B.major* (Sergent et al, 1926), and was later proved to be a distinct parasite (Zwart et al, 1968, cited by Kuttler, 1988) causing only mild infections in the UK and Northern Europe. In his 1978 report, Brocklesby proposed the existence of only four species of *Babesia* in cattle (*B.bovis, B.bigemina, B.major and B.divergens*) (Brocklesby, 1978); however, more recently, three additional species (*B.jakimovi*, *B.ovata* and *B.occultans*) have been identified by serological methods (Minami et al, 1979; Gray & de Vos, 1981; Purnell, 1981).

The geographical distribution of *Babesia* species is usually correlated with the tick vector present in the area. Thus, *B.bovis* and *B.bigemina* are the species occurring in Central and South America, Southern Europe, North Africa, Asia and Australia, transmitted by one of the species of *Boophilus* ticks, either *Boophilus microplus* or *B.annulatus*. *B.bigemina* occurs throughout Sub-Saharan Africa, transmitted by *Boophilus decoloratus* and, in some areas, by *Boophilus geigyi* or *Rhipicephalus evertsi* (de Vos, 1992). *B.bovis* only occurs in those wetter parts of Southern, Eastern and Central Africa where *B.microplus* has become established (de Vos, 1992), and in West Africa where it is presumably transmitted by *B.geigyi* (Ilemobade, 1992). *B.major* is found in Europe and North Africa transmitted by *Haemaphysalis punctata* ticks whereas *B.divergens* is found in Northern Europe transmitted by *Ixodes ricinus*. In Northern Russia the species found is *B. jakimovi* which is transmitted by *I.ricinus*, in Japan *B.ovata* is transmitted by *Haemaphysalis longicornis*, and *B.occultans* is found in South Africa where it is transmitted by the tick *Hyalomma marginatum rufipes*.

Kuttler reviewed the world-wide impact of babesiosis considering the more common *Babesia* species (Kuttler, 1988) and a summary of the bovine *Babesia* species with their dimensions, principal vectors and world distribution is reproduced in Table 2.1. Although seven species of *Babesia* infecting cattle have been described, the majority of the cattle population of the

Table 2.1 Species of *Babesia* transmitted to cattle (adapted from Kuttler, 1988).

species	morphology (length/width in μm)	principal vectors	geographic distribution
B.bovis	small (2.0 x 1.5)	*Boophilus microplus, B.annulatus, B.geigyi, Ixodes ricinus*	Central and South America, southern Europe, Africa, Asia, Australia
B.bigemina	large (4.5 x 2)	*B.microplus, B.decoloratus, B.annulatus, B.geigyi; Rhipicephalus evertsi*	same as *B.bovis*
B.major	large (2.6-3.7 x 1.5)	*Haemaphysalis punctata*	Europe and North Africa
B.divergens	small (1.5-2.0 x 0.4)	*I.ricinus*	Northern Europe
B.jakimovi	large (4.5 x 2.0)	*I.ricinus*	Siberia, Northern Russia
B.ovata	large (3.2 x 1.7)	*Haemaphysalis longicornis*	Japan
B.occultans	large (2.9 x 1.2)	*Hyalomma m.rufipes*	South Africa

world is exposed to the two most pathogenic species, *B.bovis* and *B.bigemina*, with the greatest incidence between latitudes 32° N and 32° S, where *Boophilus* ticks occur. For this reason the majority of the scientific research has been directed towards the diseases caused by these two species. Since in Brazil and most South American countries *B.bovis* and *B.bigemina* are the species affecting cattle, the present study will only review the aspects involved in *Babesia* infections caused by these two parasites.

2.1.3 Life cycle

B.bovis and *B.bigemina* develop in the vertebrate host in erythrocytes (RBC) where they multiply asexually by binary fission, with each cycle of division requiring approximately 8 hours (Mahoney, 1977) and resulting in pyriform parasites known as merozoites with a typical apical complex (Igarashi et al, 1988). The merozoites then emerge from the host cell, presumably by lysing it, and invade new uninfected erythrocytes. The process of entering starts with the attachment of the merozoite to the erythrocyte membrane, with involvement of the C3b receptor in the case of *B.bigemina* but not in the case of *B.bovis* (Igarashi et al, 1988), followed by invagination of the RBC membrane, allowing the parasite to enter with disintegration of the invaginated part of the RBC membrane. During this process the surface coat of the merozoite is lost and in the subsequent intracellular stages the parasite is covered by a single plasma membrane in direct contact with the erythrocyte cytoplasm (Igarashi et al, 1988). After penetration of an erythrocyte, the merozoite is known as a trophozoite which multiplies by binary fission or budding resulting in two merozoites.

Female ticks feeding on infected cattle ingest intraerythrocytic stages of *Babesia* which remain in the host RBC for some hours in the gut of the ticks. There a sexual cycle starts with the development of merozoites into gametocytes which divide to form four gametes. Two gametes fuse to form a zygote (Mehlhorn & Schein, 1984). The zygotes selectively infect the basophilic epithelial cells of the tick gut where multiple fission bodies are produced. These release kinetes which enter the haemolymph and invade various tissue and organs, including the developing oocytes in the ovary, to initiate cycles of multiple fission. Both *B.bovis* and *B.bigemina* are transovarially transmitted. Kinetes from the infected oocytes subsequently invade various organs in the embryo, larval, nymphal and adult ticks where further fission cycles occur.

In certain cells of the salivary glands of feeding ticks a final cycle of sporogony occurs resulting in production of sporozoites which are the forms infective for vertebrate hosts (Friedhoff, 1988). Fig 2.1 shows a diagrammatic representation of the life cycle and routes of transmission of *B.bovis* and *B.bigemina* parasites.

Fig 2.1 Diagrammatic representation of the life cycle and routes of transmission of *B.bovis* and *B.bigemina* parasites.

B.bovis and B.bigemina

1- sporozoites inoculated in saliva of feeding ticks

2- invasion of bovine erythrocytes to form trophozoites

3- binary fission of trophozoites to form merozoites

4- proliferation of parasites resulting in detectable parasitaemia and clinical disease

5- piroplasms ingested by feeding female tick develop into gametocytes (strahlenkörper) in tick gut lumen, which fuse to form zygotes

6- growth and development of zygotes into kinetes by schizogony in tick gut epithelial cells

7- migration of kinetes throughout tissues, including ovaries, of engorged female tick

8- schizogony in developing oocytes

9- migration of kinetes throughout larval tissues

B.bovis only

10- entry of kinetes into larval salivary gland cells and subsequent sporogony during larval feed

B.bigemina only

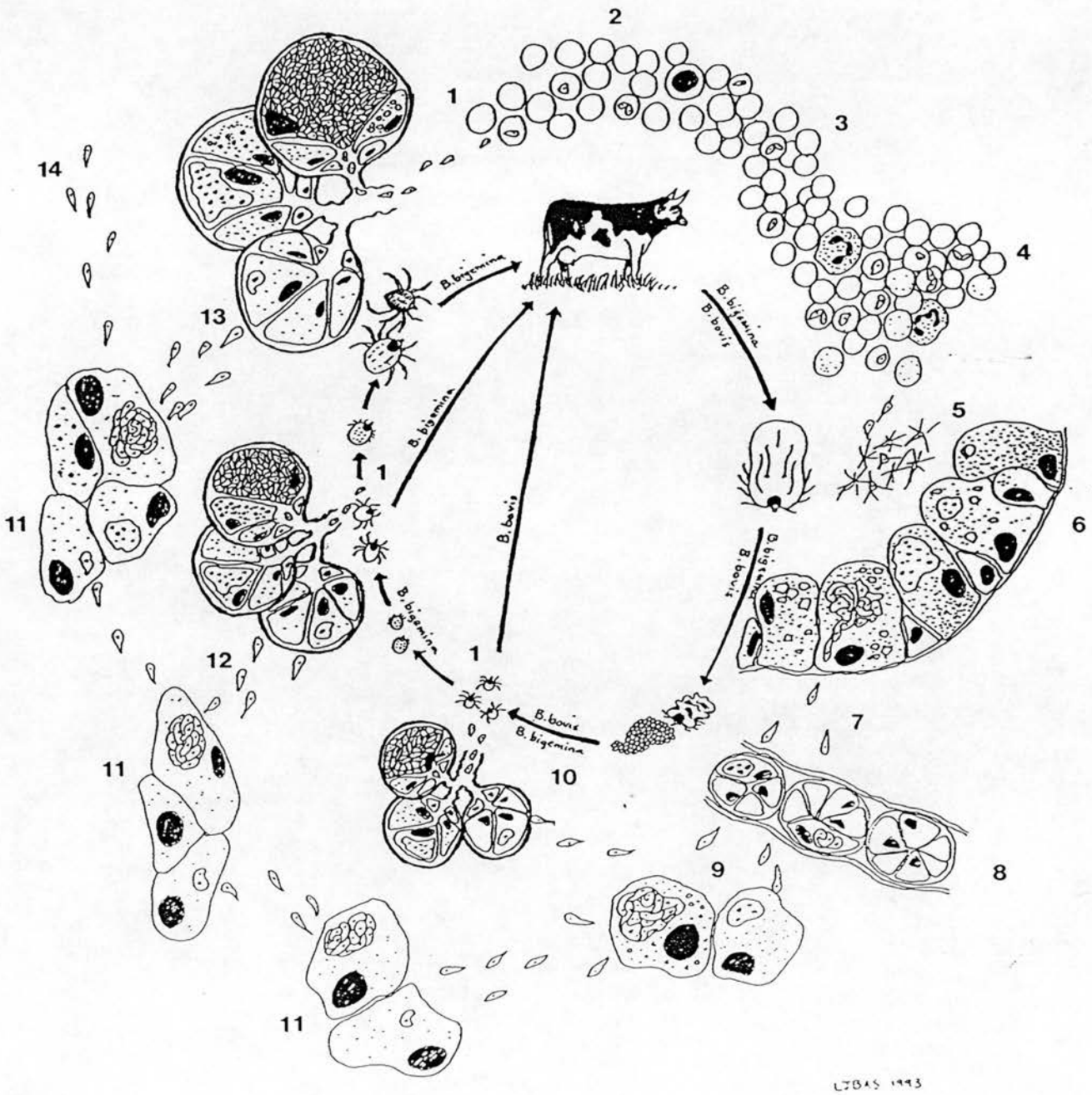
11- cycles of schizogony followed by kinete migration in larval/nymphal tissues

12- entry of kinetes into nymphal salivary gland cells and subsequent sporogony during nymphal feed

13- entry of kinetes into adult salivary gland cells and subsequent sporogony during adult feed

14- further cycles of sporogony in feeding/engorged female resulting in vertical transmission through infection of developing oocytes

Fig 2.1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The life cycles of the two species have distinct characteristics. *B.bovis* is transmitted only by the larval stage of *B.microplus* or *B.annulatus* which then loses the infection, and vertical transmission does not occur (Mahoney & Mirre, 1979). The infection of the tick population is therefore based only on alimentary infection of the engorging females which will result in infection of a low proportion of their eggs. *B.bigemina*, on the other hand, is transmitted by feeding *B.microplus* and *B.decoloratus* nymphs, females and males, though transmission by females and males of *B.annulatus* has not been demonstrated (Friedhoff, 1988). In addition, persistence of infection throughout a complete life cycle and two further ecdyses occurs in *B.microplus* ticks, since the adults resulting from a second generation of infected eggs are still able to transmit the infection (Friedhoff, 1988). These particular characteristics result in higher rates of *B.bigemina* infection in ticks.

2.1.4 *In vitro* cultivation

The *in vitro* cultivation of *B.bovis* in bovine erythrocytes was first described by Erp and co-workers as a short term system with slow stirring of cultures in spinner flasks (Erp et al, 1978). Later, the same group of researchers examined several variables in culture conditions affecting parasite growth, including medium composition, pH, gas tension and storage of serum and RBC (Erp et al, 1980). They concluded that amongst these the pH markedly influenced growth rates and found that the optimum culture pH was 7.0. Levy and Ristic (1980) improved this culture system to overcome some of the limitations of the original technique, such as the lengthy manipulations necessary and slow growth rates obtained, by culturing the parasite in a microaerophilous stationary phase (MASP), similar to the method developed for *Plasmodium falciparum* (Trager & Jensen, 1976). The original MASP system consisted of a suspension of infected erythrocytes in medium M199 supplemented with 15 mM HEPES and 40% normal bovine serum (NBS) with a PCV of 5 to 10% and incubated, at a ratio of 0.62 ml to 1cm^2 of culture area, at 37^{o}C under an atmosphere of 5% CO_2 in air (Levy & Ristic, 1980). The overlying medium was replaced with fresh culture medium at 24-hour intervals with subcultures being carried out every 48 to 72 hours by diluting the culture suspension 2 or 3-fold with fresh uninfected RBC suspension.

The MASP system has facilitated studies on many aspects of *B.bovis* including parasite ultrastructure (Aikawa et al, 1985) and metabolism (Conrad, 1986; Matias et al, 1990), drug sensitivity assays (Jasmer & Goff, 1989; Brockelman & Tan-aryia, 1991), the role of serum factors in parasite growth (Levy et al, 1986; Tambrallo et al, 1992), immunodiagnosis and immunoprophylaxis using either parasite soluble exoantigens released into culture supernatants (Montenegro-James et al, 1981; 1983) or structural components

(Goff et al, 1988; McElwain et al, 1988), and genetic and molecular characterisation of parasite isolates (Tripp et al, 1989; Dalrymple, 1990; Brown et al, 1993).

Short term cultivation of *B.bigemina* has also been developed and was first described by Timms (1980) using a method similar to that developed for human malaria parasites (Trager & Jensen, 1976), however the infected erythrocytes could not been maintained beyond 96 hours. Vega and co-workers (Vega et al, 1985a) optimised the technique, achieving a continuous cultivation system by washing the RBC in a phosphate-buffered saline solution (containing $CaCl_2$, KCl, $MgSO_4$ and dextrose) and culturing them in a stationary phase similar to that developed for *B.bovis*, as a 5 to 10% RBC suspension in M199 supplemented with 20 to 50% NBS at a depth of 4 mm in a 5% CO_2, 2% O_2, 93% N_2 atmosphere. More recently, Jorgensen and co-workers described a suspension culture system, using a PCV of 13% and gas mixtures of 2%-3% O_2, 5% CO_2 and 92-93% N_2, which has proven more efficient than the MASP system and is able to satisfy the present demand for *B.bigemina* live vaccine in Australia (Jorgensen et al, 1992).

Following the development of a continuous *in vitro* culture system for *B.bigemina*, cultured parasites have been used to study many aspects related to the parasite including cryopreservation (Vega et al, 1985b), drug susceptibility assays (Brockelman & Tan-ariya, 1991), cloning (Vega et al, 1986a), concentration of erythrocytic stages (Vega et al, 1986b; Figueroa et al, 1990a) and parasite characterisation (McElwain et al, 1987; 1991; Montenegro-James et al, 1987; Buening et al, 1990; Figueroa et al, 1990b; Mishra et al, 1991; Tambrallo et al, 1992). Although a significant amount of work has been performed using *B.bigemina* cultured parasites, only a limited number of isolates have been adapted to continuous *in vitro* culture and the system has thus not advanced as far as that of *B.bovis*.

2.2 THE DISEASES

2.2.1 Clinical signs and pathogenesis

A wide variety of clinical signs caused by *Babesia* infections can be observed in cattle with two important factors affecting the severity of infections. The first is the virulence of the species and strain, with small babesias such as *B.bovis* being highly pathogenic, while large babesias such as *B.bigemina* are generally less virulent, although variable virulence between strains of *B.bigemina* has been reported with African and Israeli strains being highly virulent while the Australian strains are of low to moderate virulence (Wright & Goodger, 1988; Pipano, personal communication). The second factor is related to the susceptibility of the host which is dependent on its age, breed, physiological and immunological status, and environmental stress (Mahoney, 1977). Young animals are reported to be more resistant to *B.bovis* infections than adult ones independent of the presence of colostral antibodies (Riek, 1963) and *Bos indicus* animals have been shown to be less susceptible than *Bos taurus* breeds (Johnston et al, 1978). Splenectomy can exacerbate symptoms caused by less pathogenic species such as *B.bigemina* and result in increased parasitaemias of *B.bovis*, although splenectomised calves infected with *B.bovis* are not necessarily more severely affected than intact calves (Wright & Goodger, 1988). The diseases are usually associated with anaemia, fever, dehydration and enlargement of the spleen. *B.bovis* parasites show a predilection for capillaries in the brain and kidney which result in damage of tissue cells by depletion of their oxygen supply and therefore a more severe disease, whereas *B.bigemina* is distributed throughout the bloodstream. Both infections are accompanied by lysis of erythrocytes and haemoglobinaemia/haemoglobinuria followed by jaundice, the latter being most commonly observed in *B.bigemina* infections.

The incubation period after tick infestation is usually between 8 and 16 days with infection by *B.bovis* appearing first due to the transmission by the larval stage, and *B.bigemina* appearing a few days later due to the transmission by the nymphal stage. Rectal temperature rises in parallel with the increase in parasitaemia reaching a maximum of 41.0 to 41.5^{o}C within two or three days and the animal becomes listless, anorectic and weak. Haemoglobinuria occurs followed by jaundice, particularly in *B.bigemina* infections, and signs of cerebral damage such as paddling of limbs, ataxia, and coma are often seen in infection with *B.bovis*. Mortality is usually high with *B.bovis* infections (Mahoney, 1977; Kreier & Baker, 1987).

Recovery from the acute infection is accompanied by clearance of parasites from blood with the animals becoming immune and not normally showing recrudescence of disease.

The pathogenesis of babesiosis is complex with a multisystemic involvement, and appears to result from the release of pharmacologically active substances and destruction of

erythrocytes. Severe coagulation disturbance related to fibrinogen metabolism results in hypercoagulability and rapid formation of cryoprecipitates in plasma of animals infected with *B.bovis* (Goodger, 1975; Goodger & Wright, 1979). Goodger and co-workers also reported that the *B.bovis* infected erythrocytes have an enhancing effect on the procoagulant activity and that the coagulation time of normal bovine plasma was reduced by between 33 and 66% in the presence of infected erythrocytes, depending on the strain of parasite (Goodger et al, 1987b). These authors suggested that disturbance of coagulation associated with an affinity of fibrinogen for infected cells might be the explanation for the capillary stasis often observed in *B.bovis* infections (Wright & Goodger, 1988), which appears to result from the activation of the coagulation system *in situ* causing the strand-like formations reported by Wright (1972).

During acute infection with virulent strains of *B.bovis* there is also an increase in circulating activated kallikrein and a large decrease in plasma kininogen and subsequent production of bradykinin and other vasoactive agents (Wright, 1973a, 1977), which might be associated with falls in systolic blood pressure and correlated with haematocrit reduction (Wright & Goodger, 1988). In addition, thrombocytopenia occurs as a result of a rise in the levels of histamine and 5-HT (Wright, 1978), which potentiate the effect of kinins and kallikrein (Mizuta, 1970).

Stasis induced by aggregated *B.bovis* infected erythrocytes in the microvascular system has been reported to be the most deleterious lesion (Wright & Goodger, 1988), resulting from infected cells adhering to each other and to the endothelium of the brain, kidney and skeletal muscle (Wright, 1972, 1973a; Wright et al, 1979). The mechanism by which the stasis occurs is probably multifactorial and cascadial involving: *i*) the endothelium, possibly by an interaction between endothelial fibrin and erythrocytic fibrinogen (Goodger, 1978); *ii*) changes in the erythrocyte membrane, perhaps due to the presence of babesial antigens on the surface of the infected erythrocytes (Goodger et al, 1980) or by resultant increase in viscosity and decrease in deformability of the erythrocytes due to lipid peroxidation (Commins et al, 1988) and *iii*) increased host plasma concentrations of fibrinogen, soluble fibrin and fibrinogen-fibrin complexes resulting in increased plasma viscosity (Goodger & Wright, 1979; Goodger et al, 1980).

2.2.2 Diagnosis

Microscopic examination of thin or thick blood smears stained with Giemsa is the usual diagnostic procedure for confirmation of a case of acute babesiosis. For diagnosis of *B.bovis*,

thin smears are usually made from peripheral capillary blood and, if the animal is dead, from kidney, brain, spleen and liver, where infected RBC tend to concentrate.

However, due to the difficulty of detecting parasites in blood smears from chronically infected animals, direct microscopic examination is not applied for detection of carriers. The recent advent of nucleic acid probes for *B.bovis* (McLaughlin et al, 1986) and *B.bigemina* (Buening et al, 1990) and the use of the polymerase chain reaction (PCR) to enhance their sensitivity have provided researchers with the most sensitive and specific assays for detection of infection in carriers which are expected to become routine tests within the near future (Palmer, 1992).

On the other hand, serological tests can be used as an indirect indication of whether or not an animal has previously been infected with a particular parasite, and still remain the most widely-used procedures for epidemiological surveys . The indirect diagnosis of infection by detection of specific antibodies is appropriate for epidemiological studies of bovine babesiosis, since animals that recover from acute infections become carriers and remain immune for at least 4 years in the case of *B.bovis,* and for 2-3 years in the case of *B.bigemina* infections (Mahoney et al, 1973, 1979). Evaluation of the serological status of the animals is also useful for monitoring vaccination and chemotherapeutic programmes.

An ideal serological test should not only have high sensitivity and specificity, but it should also be easy to perform, inexpensive and provide reliable results.

A large number of serological tests have been developed for detection of antibodies to *Babesia* parasites with their sensitivity (indicated by the number of false negative results) and specificity (indicated by the number of false positive results) depending on the antigen preparation used. The different serological tests that have been applied for detection of anti-*Babesia* antibodies have been extensively reviewed by Todorovic and Carson (1981), Weiland and Reiter (1988) and Wright (1990). These include complement fixation (CF), indirect haemagglutination (IHA), latex agglutination, indirect fluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) and a modification of this (slide ELISA), and radioimmunoassay (RIA). However, the IFAT and ELISA are the most commonly used.

The source of antigen is either whole blood from infected animals collected during ascending parasitaemia, or, with the development of *in vitro* culture systems for *B.bovis* (Levy & Ristic, 1980) and *B.bigemina* (Vega et al, 1985a), cultured parasites can also be used for preparation of antigens for serological tests (Jones & Conrad, 1983; Woodford et al, 1990).

2.2.2.1 IFAT

For the IFAT, whole infected erythrocytes are used to coat microscope slides, which can be stored at low temperatures (-20^{o}C), and are used as antigen for detection of antibodies present in test sera. The test is based on the indirect detection of the parasite antigen - specific antibody complex in bovine serum by its reaction with a second anti-species immunoglobulin which has been previously conjugated to fluorescein isothiocyanate. Although the IFAT is sensitive and easy to perform, interpretation is subjective and background fluorescence due to non-specific binding of antibodies to host contaminants can be confused with low titre positive serum samples (Callow et al, 1974). This, in association with the limited number of samples that each operator can examine in one day and the requirement for ultra-violet microscopy constitute the major limitations of the test. Cross-reactivity between *B.bovis* and *B.bigemina* is usually observed, particularly with *B.bigemina* antigens (Bessenger & Schoeman, 1983; Wright, 1990).

The use of a fluorescent antibody test was first described by Ristic and co-workers who used it to study the development of *B.caballi* and *B.equi* in horse erythrocytes and to detect circulating anti-*Babesia* antibodies in a one-step inhibition test (Ristic et al, 1964; Ristic & Sibinovic, 1964).

In 1968, Ross and Lohr described an IFAT for detection of anti-*B.bigemina* antibodies in bovine serum by which maximum titres of 1: 1280 were observed at 21 days after infection (Ross & Lohr, 1968).

Ross and Lohr (1970) used the IFAT to study the persistence of colostral antibodies to *B.bigemina* in calves and found higher titres in calves up to 1 month of age than in their dams, which persisted for 17 weeks after birth. Based on these results the IFAT is not considered an appropriate test for determination of *Babesia* infection rate in young calves.

Brocklesby and co-workers, using the IFAT to examine bovine serum, identified as *B.major* a large *Babesia* derived from *H. punctata* ticks collected in two separate localities in South-East England (Brocklesby et al, 1971).

In an attempt to facilitate epidemiological studies, Burridge and co-workers proposed the use of blood dried on filter paper as a source of antibodies to be tested by IFAT (Burridge et al, 1973). This technique was evaluated by Todorivic and Garcia (1978) for detection of antibodies to *B.bovis* and *B.bigemina* in blood from Colombian cattle under laboratory and field conditions. A high correlation was found between the results obtained with serum and dried blood, and the authors suggested that samples on filter paper could be practical for field use in epidemiological studies since they were easy to handle and could be stored at various temperatures.

Comparisons between the IFAT and the CF test have shown that IFAT is more sensitive, detecting *B.bovis* antibodies an average of 2 weeks earlier than CF and *B.bigemina* antibodies 1.5 weeks earlier (Todorovic & Long, 1976). In that study IFAT titres rose to levels of between 1:640 and 1:51,220 compared with CF titres ranging from trace to 1:80. A similar comparison was carried out by Kuttler and co-workers, who found that both tests were effective in detecting *B.bigemina* antibodies during the first 84 days of infection, both showing a similar linear regression in serum titres; however after 94 days post-infection the IFAT titre regression began to plateau while the CF titres decreased below background levels in 60% of the samples tested (Kuttler et al, 1977).

Due to its simplicity and high sensitivity, the IFAT has been widely used in a large number of studies, namely on antigenic relationships between *Babesia* species, and for epidemiological surveys all over the world, and it still remains the test most used for serological diagnosis of bovine babesiosis (Leeflang & Perie, 1972; Rodriguez et al, 1979; Bessenger & Schoeman, 1983; Madruga et al, 1984; Miller et al, 1984).

2.2.2.2 ELISA

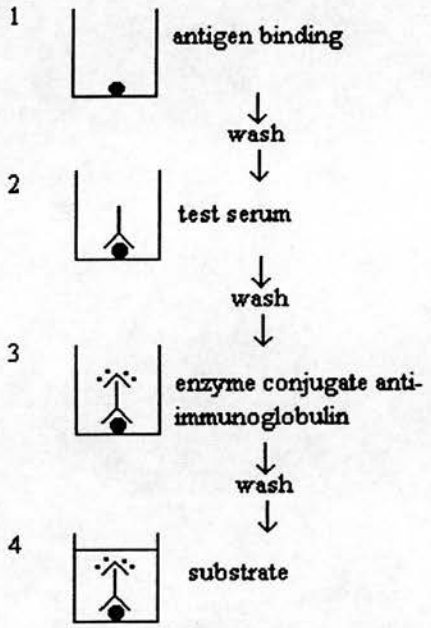
The ELISA was described as a very sensitive and economical assay for detection of antibodies by Engvall and Perlmann (1972). Tests were carried out in plastic tubes but these were inconvenient for large scale use. The test was then adapted for performance in disposable polystyrene microhaemagglutination plates (Voller et al, 1976), which are now generally used as the antigen/antibody carrier. It was first shown to be suitable for detection of *Babesia* antibodies by Purnell and co-workers (Purnell et al, 1976). The ELISA involves three stages, first a reaction between the antigen and corresponding antibodies, followed by the detection of this reaction by the use of anti-species immunoglobulin conjugated to an enzyme. In the last stage of the assay the respective substrate for the enzyme is added and the amount of antibody bound in the first reaction is indicated by the degree of substrate degradation, which can be quantitatively measured on a spectrophotometer.

A number of alternative assay systems can be used for the detection and measurement of antibodies or circulating antigens and immune complexes using antigen or antibody solid phase coating principles. The various methods for ELISA have been reviewed by Catty and Raykundalia (1989). However, two main types of systems are used to detect antibodies, namely the indirect ELISA and the indirect double antibody sandwich ELISA and these are represented diagrammatically in Fig 2.2.

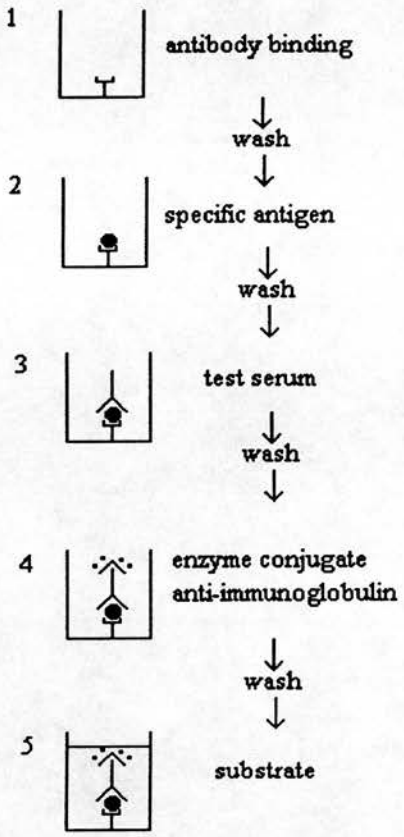
So far, the assays developed for *B.bovis* are based on the indirect detection of antibodies (Barry et al, 1982; Waltisbuhl et al, 1987; Bose et al, 1990).

Fig 2.2 Schematic representation of the different stages involved in the indirect ELISA (A) and the indirect double antibody sandwich ELISA (B) (adapted from Sanchez-Vizcaino and Cambra Alvarez, 1987).

Fig 2.2


1
antigen binding
wash
2
test serum
wash
3
enzyme conjugate anti-immunoglobulin
wash
4
substrate

(A)


1
antibody binding
wash
2
specific antigen
wash
3
test serum
wash
4
enzyme conjugate anti-immunoglobulin
wash
5
substrate

(B)

In any of the ELISA systems there is a need to standardise all stages of the test by determining the optimal antigen, antibody and conjugate dilutions which give the greatest discrimination between positive and negative reference sera in a checkerboard titration (Voller et al, 1979). The interpretation of ELISA results is also a subject which deserves great attention. The determination of the positive-negative discrimination levels is known as the "cut-off value" and can be calculated in different ways, as reviewed by Tijssen (1985): *i*) by the mean absorbance of the negative control sera multiplied two or three times, *ii*) by the mean of negative control absorbance plus two or three standard deviations, *iii*) by an arbitrary value, usually between 0.15 and 0.20 absorbance. However, as demonstrated by Beier and co-workers, positivity rates in an ELISA for determination of malaria sporozoite infection rate in *Anopheles* mosquito populations could be significantly over-estimated depending on the absorbance cut-off method applied for negative controls (Bier et al, 1988).

The original ELISA test developed for detection of *B.bovis* antibodies used as antigen infected blood with 20% parasitaemia from which infected erythrocytes were concentrated by a procedure of lysis with 0.425% potassium chloride, followed by removal of leucocytes using CF11 cellulose, and lysis of infected cells to remove haemoglobin (Barry et al, 1982). The conjugate used for detection of reactions was anti-IgG-alkaline phosphatase. Comparison with IFAT showed more than 95% agreement between the two tests. The ELISA was shown to be specific for *B.bovis* by the lack of significant cross reactivity with *B.bigemina*, *Anaplasma centrale* and *Theileria* positive sera and to be more sensitive than the IFAT by titration of positive sera. Antibodies in experimentally infected cattle were detected on day 14 after infection and lasted for at least 268 days.

A comparison between the ELISA, IFAT and CF for detection of antibodies in sera of cattle experimentally infected with *B.divergens* and *B.major* was carried out by Bidwell and co-workers, who reported that CF detected antibodies earlier but that the CF titres were consistently lower than those obtained with IFAT and ELISA. These authors found agreement between the results from IFAT and ELISA. Nevertheless they suggested that the ELISA may be preferable due to the fact that it is less subject to operator error, and therefore more accurate, and also larger numbers of samples can be examined (Bidwell et al, 1978).

In an attempt to make the ELISA technique more economical and technically simpler, Kung'u and Goodger developed a slide enzyme-linked immunosorbent assay (SELISA) for detection of *B.bovis* antibodies, in which smears made from infected blood, fixed in acetone and stored at -70^{o}C, were used as the source of antigen (Kung'u & Goodger, 1990). The test was performed in a manner similar to IFAT but using horseradish peroxidase conjugate and 4-chloro-1-naphthol as the substrate for detection of reactions. Monoclonal antibodies were also tested to demonstrate the cellular specificity of the test. The slides were examined using a

light microscope and positive reactions were indicated by bluish-purple staining of the *B.bovis* infected erythrocytes or the parasites, or both, whereas in negative reactions no staining of either the infected cells or parasites occurred. The SELISA was found to be very sensitive and detected antibodies to *B.bovis* for at least 36 months. Due to precise cellular specificity obtained with different monoclonal antibodies, the test may be suitable for screening of hybridomas (Kung'u & Goodger, 1990).

In cases where provision of parasites from infected animals is a limitation, the *in vitro* culture system for *B.bovis* has been successfully used to provide infected erythrocytes as a source of antigen for ELISA (Hadrill et al, 1990; Woodford et al, 1990). In these studies leucocytes were removed from culture suspensions with parasitaemias between 4 and 10%, by column chromatography using cellulose powder (CF11) (Melrose and Brown, 1979). The infected erythrocytes, which are osmotically less fragile than uninfected erythrocytes, were concentrated by selective hypotonic lysis with potassium chloride. Concentrated *B.bovis* infected cells were then disrupted by sonication and the resulting soluble extract was used as crude antigen. Some degree of cross-reactivity with *B.bigemina* sera was detected but it was considered not to interfere with the interpretation of the results from the assay.

As the ELISA has proved to be a highly sensitive test, quick to perform and suitable for surveys in which a large of samples have to be processed, attempts have been made to improve it particularly with regard to the use as antigen of purified fractions of the parasite instead of crude extracts. Contamination of parasite extracts with host components, particularly erythrocyte stroma and membranes, results in binding of non-specific antibodies which result in background absorbance values in negative sera. Attempts to reduce non-specific background absorbance have been made based on the use of a blocking step before the addition of test sera, using reagents such as detergents and proteins, which occupy non-specific reactive sites (Spencer, 1988). In addition, non-specific binding due to presence of anti-erythrocyte antibodies is also observed in sera from animals which had previously been inoculated with infected erythrocytes; this can be reduced by pre-adsorption of the sera with normal bovine erythrocytes before the test (Waltisbuhl et al, 1987).

Purified fractions of a lysate of *B.bovis* infected erythrocytes, obtained after gel filtration using Sephadex G200, have been used as antigens in ELISA (Waltisbuhl et al, 1987). One of the fractions obtained (fraction II) was selected for the development of the test since it was found to be the most sensitive with *B.bovis* antisera; however it showed some cross-reactivity when tested against *Anaplasma marginale* sera. In the same study four different enzyme conjugates and their respective substrates were tested to determine the most sensitive system for detection of *B.bovis* antibodies, and horseradish peroxidase was found to be the most suitable.

More recently, a recombinant form of an antigen from *B.bovis* (11C5), which had been shown to induce immunity in cattle, was found to be sensitive and specific when used as antigen in ELISA, although lower sensitivity was observed with some positive serum samples (Bose et al, 1990). These authors suggested that perhaps a 'cocktail' of two or more recombinant antigens was needed to improve the sensitivity of the test. The major advantages of recombinant proteins as a source of antigens would be the elimination of variation between antigen batches, and also the reduction of non-specificity due to host contamination, which are limiting factors for the use of crude antigen. In the same study a crude antigen preparation was used and it was reported that the use of IgG-specific monoclonal antibody conjugate instead of conventional conjugates against bovine IgG virtually abolished non-specific reactions. The assay was also optimised when a blocking step using PBS containing 2% horse serum was included before the reaction with test sera (Bose et al, 1990).

The same synthetic *B.bovis* antigen (11C5) has been shown to be suitable for use as antigen in an ELISA for the diagnosis of *B.ovis* in Turkey and successfully replaced the conventional crude preparation (Duzgun et al, 1991).

A kit for carrying out an indirect ELISA for the detection of *B.bovis* antibodies has been supplied by the Joint FAO/IAEA Division, Vienna, for serological surveys in some Latin America countries. Recently, the kit was evaluated in Uruguay for use in serological surveys (Cardozo et al, 1990). The test showed a sensitivity of around 98% and specificity of 97%, although significant variability in plate to plate absorbance values was observed, and no cross-reactions were seen when a panel of experimental sera against *B.bigemina* and *A.marginale* were tested. The FAO/IAEA kit has also been used for a serological survey in Mexican farms, where prevalence rates were found to be high enough to result in enzootic stability (Vazquez et al, 1990).

Recent modifications in ELISA systems include solid phase reactions on micropegs, dip-sticks and beads, and the so called 'dot-ELISA' assays on protein-binding membranes such as nitrocellulose, in which very small amounts of sample can be used (Catty & Raykundalia, 1989). A dot-ELISA system which could be read visually and gave results within three hours has been developed for use in serodiagnosis of bovine anaplasmosis (Montenegro-James et al, 1990).

The use of ELISA has also been reported for the detection of culture-derived exoantigens of *B.bovis*, in which purified anti-*B.bovis* IgG isolated from immune cattle was used as a capture antibody and as an enzyme-conjugated recognising antibody (Montealegre et al, 1987). In that study 24-hour cultures showed the greatest concentration of exoantigens and the assay was sensitive for detection of differences in species-specific antigenic activity

between some *B.bovis* isolates. However cross-reactions with *B.bigemina* antigens were observed.

Specific ELISA systems for serodiagnosis of *B.bigemina* are not as advanced as the *B.bovis* system, basically due to numerous difficulties encountered in the preparation of soluble extracts from this parasite for use as antigen. These limitations are related to the concentration of infected erythrocytes from blood, isolation of intact parasites from RBC, extraction of antigenic components from parasites and contamination with host material. In addition, antigen preparations from *B.bigemina* usually cross-react with *B.bovis* antisera. In an attempt to overcome most of these limitations, O'Donoghue and co-workers developed a technique for purification of *B.bigemina* parasites from blood for use in an enzyme immunoassay (O'Donoghue et al, 1985). The method was based on a procedure of controlled lysis with hypotonic saline (0.06 M) by which the merozoites were freed from the erythrocytes, and were then disrupted by sonication. The soluble extract was then subjected to gel filtration chromatography using a Sephadex G-200 column that resulted in purification of two antigenic fractions, which were used to monitor by ELISA the antibody response of an infected calf. The tests using the two semi-defined fractions of *B.bigemina* appeared to be sensitive, however no information concerning their specificity was given.

Schuntner and Wright also developed a serological test for *B.bigemina* using a radioimmunoassay, in which a crude extract was prepared by sonication of infected blood. The antibody response to the portion of the extract remaining after depletion by acidification, followed by a solvent extraction, was subtracted from the response to the initial crude preparation giving the net specific *B.bigemina* response (Schuntner & Wright, 1989). The assay gave < 2% of false negative reactions, 2% false positives with negative sera, 4% false positives with *B.bovis* sera and no false positives with *A.marginale* sera, and the specific anti-*B.bigemina* antibodies were detected for at least 10 months after infection. Although the RIA seems to be both sensitive and specific, it is a sophisticated test requiring a well-equipped laboratory and it is unlikely that it could be widely used for field surveys in the near future.

2.2.3 Epidemiology

The epidemiology of bovine babesiosis is a difficult subject since complex interrelationships between the vector and the host are involved and therefore detailed analysis of the various components of the tick-*Babesia*-cattle system must be considered.

The vector component *Boophilus* (in the case of *B.bovis* and *B.bigemina* infections) is a one-host tick considered to be specific to cattle, with the infestation of other hosts having no

epidemiological importance. According to Alonso and co-workers, amongst the components of the system, *Babesia* is the weakest, since it depends on ticks and cattle for maintenance and propagation, followed by *Boophilus*, which depends on cattle and finally the cattle which do not depend on either, and act as a support for the other two components (Alonso et al, 1992).

Relating to the *Babesia* parasite the most significant factors are the percentage of mortality of tick larvae and the ability to overcome immunological reactions from the host. Mortality of larvae is reported to be between 74 and 90% and can be even higher when considering *Boophilus*-resistant cattle (Sutherst & Utech, 1981). Climatic factors may also affect oviposition, development of eggs and mortality of larvae (Harley, 1966) and therefore the infection rate of ticks must be high to overcome all these barriers.

The epidemiology of bovine babesiosis has been extensively studied in Australia over the past two decades by Mahoney and co-workers (Mahoney, 1969; 1977; Mahoney et al, 1973). The numerical relationships between the steps in the sequence of events during infection of the vertebrate host and transmission of the parasite from one host to another must be calculated in order to understand the complex interactions involved and to design effective control measures.

According to Mahoney (1977) the probability of host infection is an important parameter and reflects the stability of the system; if this probability is high enough, the young animals receive an initial infection before their passive immunity wanes, the situation is stable and clinical cases do not occur, although *Babesia* organisms are widespread in the environment. On the other hand, if the probability of host infection is low, a proportion of the animals do not receive initial infection during the passive immunity period and therefore there is a risk of them becoming clinically ill thereafter; this characterises an unstable situation. The probability of host infection is measured by the "inoculation rate" which represents the probability of infection of any member of the host population during 1 day, and is a product of the number of bites received from the vector by each host per day (m), the proportion of the vector population carrying infective forms of *Babesia* (a), and the proportion of infected bites that successfully infect the host (b). Thus, estimation of the inoculation rate involves counts of ticks on the bovine host and microscopic examination of tick smears for determination of babesial infection rate, and is calculated by the formula:

$$\text{Inoculation rate (h)} = mab \qquad (1)$$

The rate at which young animals acquire the infection is also related to the inoculation rate and is given by the equation:

$$I = 1 - e^{-ht} \qquad (2)$$

where I = proportion of animals infected (measured by a serological test), h = inoculation rate, t= average age of the animals in days, and e is the natural logarithm.

Using equation 2, the inoculation rate for a determined age group can be calculated if the proportion of infected animals (I) is known.

The range of values of the inoculation rate depends on the length of time after birth for which the passively acquired protection is effective. If this period has been defined, the critical value for an inoculation rate which will result in enzootic stability can be calculated by substituting that value for t in equation 2. Inoculation rate values equal to or above this critical level will indicate endemic stability, whereas inoculation rate values below the critical level indicate instability, with risk of disease.

The minimum number of ticks infesting the vertebrate host which will ensure stability can also be calculated if the infection rate of the ticks is known, using equation 1. Mahoney (1977) reported that at least 12 larvae of *B.microplus* per animal (*Bos taurus*) per day were required to result in a critical inoculation rate of 0.005 to maintain stability for *B.bovis*. The same values have been used in epidemiological studies where no information about infection rates of ticks are available (Vazquez et al, 1990).

When inoculation rates fall below the critical level for stability, there is a range of values which will produce a high incidence of clinical disease in animals which did not receive infection before passive immunity waned (zone of maximum risk); on the other hand, if the reduction in inoculation rate falls too low, a large proportion of the population may never be exposed to infection and the incidence of clinical disease might be very low. Knowledge and understanding of the epizootiology of bovine babesiosis is an essential prerequisite to proposal of effective control measures, which will vary in each situation.

It is important to emphasise that the genetic composition of cattle substantially affects the estimation of inoculation rates, as discussed by Alonso and co-workers (Alonso et al, 1992). *B.microplus* infestation is inversely proportional to the proportion of *Bos indicus* genes (Lemos et al, 1985; Guglielmone et al, 1990) and parasitaemias of *B.bovis* are also generally lower in *Bos indicus* than in *Bos taurus* cattle (Aguirre et al, 1990), and therefore lower infection rates are observed in ticks feeding on *Bos indicus* breeds. Guglielmone and co-workers have reported infection rates of 28%, 23% and 5% respectively in female ticks fed on Creole (*Bos taurus*), Hereford (*Bos taurus*) and Nelore (*Bos indicus*) cattle (Guglielmone et al, 1989); these differences will affect the epizootiology of babesiosis.

In addition to the inoculation rate, host immunity will affect the stability of bovine babesiosis. There are several aspects which affect host immunity and which should be taken into account.

First of all, there is age resistance. Although in some situations babesiosis is considered to be more pathogenic in calves (Madruga et al, 1984), animals below 9 months of age appear to have a natural protection, possible related to the presence of a factor responsible for inhibition of parasite growth (Levy et al, 1982); in addition, colostral antibodies have been detected for many months after birth giving passive protection to calves born of immune dams (Ross & Lohr, 1970). The second factor is the duration of immunity, which in both *B.bovis* and *B.bigemina* infections is retained for periods of at least four years, regardless of the infection status (Mahoney, 1974). Lastly, immunological differences between isolates may also have an effect in the maintenance of stability depending on the frequency with which cross infection between isolates causes recurrence of clinical disease (Mahoney, 1974).

Another important concept that involves transmission is the basic reproduction rate, which is a hypothetical number representing the secondary cases of a disease disseminated by a single primary case in a non-immune individual in an environment where neither the vertebrate host nor the vector population were previously infected. The basic reproduction rate is affected by: *i*) the duration of infectivity for ticks of a primary case, *ii*) the number of female adult ticks dropped per day, *iii*) the number of larvae produced per tick and *iv*) the proportion of larvae that bite a new host. If it is equal or greater than 1 the disease should persist, if it lower than 1 the disease should disappear. The basic reproduction rate can be calculated by the formula:

$$2\,d\,n\,a$$

where d = duration of infectivity in days, n = number of female ticks dropped per day and a = babesial infection rate of all the progeny (Mahoney, 1977). A critical level of tick infestation (n) for maintenance of *Babesia* organisms in the environment can be calculated by the equation

$$2\,d\,n\,a = 1.$$

The great limitation of this equation seems to be related to the estimation of babesial infection in ticks, which takes place during the last 24 hours of female feeding on cattle (Callow, 1968). *Babesia* kinetes are easier to detect in haemolymph of engorged females than in larvae. However Mahoney and Mirre (1971) considered that this technique underestimated the number of infected females. Moreover, the relationship between the number of kinetes seen in haemolymph from female ticks and percentage of infective larvae is not known. Differences between calculated and observed rates of vector infection have been reported, implying that calculated values are not always reliable (Mahoney et al, 1981b).

There is evidence that the exposure of larvae at 14^{o}C for three to five weeks increased their infection rate with *B.bovis* and *B.bigemina* (Dalgliesh & Stewart, 1982). The influence of

climatic factors has also been reported on the longevity of larvae and on the annual number of generations of *B.microplus* (Sutherst & Utech, 1981), and on the transovarial transmission of *Babesia* (Riek, 1964). Another aspect involved in the *Boophilus-Babesia* system is the pathological effect of *Babesia* on ticks, which has been reported with *B.bovis* and *B.bigemina* experimental infections (Guglielmone et al, 1989). However, the low reliability of calculated values of vector infections (a) does not necessarily invalidate the estimation of the inoculation rates (h) by the proportion of animals infected (I) for a particular age group of animals (t).

On top of all the biological parameters which influence the interactions involved in the *Babesia*-tick-cattle system, comes husbandry/management, which will also interfere with the system. Thus, actions such as selection or introduction of European breeds into herds of indigeneous cattle or vice versa can lead to changes in the enzootic stability of a herd; the use of acaricides with long residual periods may prevent transmission of *Babesia* at levels high enough to maintain stability; irrigation of pastures during dry seasons may result in an increase in the number of tick generations during the year, and so on.

The relationships between *B.bovis* and its tick vector (*Boophilus*) have been studied by computer simulation with an interactive BASIC program by Smith (1983), who derived the major independent variables (host-finding success, feeding success, filial infection rate, recovery rate) from published laboratory and field data, and calculated, during successive tick generations, values for dependent variables (tick burden, inoculation rate, tick and bovine infection rates). The simulation was used to predict the effects of different strategies of disease control. Daily infestation rates of 8 to 9 engorged ticks were found to be optimum to maintain immunity to babesiosis without significant physiological stress to the bovine host, whereas disease outbreaks were associated with daily infestations of between 2 and 8 engorged females.

More recent studies on simulation modelling include the use of climatic matching programmes, such as "Climex" (Sutherst & Maywald, 1985), and programmes in which all of the features of the tick model (management of the hosts, for instance) are incorporated, such as the TICK2 (Dallwitz, 1987).

2.2.4 Control

Bovine babesiosis is widespread in tropical and subtropical regions where, due to favourable climatic conditions, the *Boophilus* tick vector can complete several generations throughout the year. In these conditions the high rate of transmission results in most cattle becoming infected at a young age, leading to enzootic stability.

The epizootiological principles proposed by Mahoney (discussed in section 2.2.3) can be applied to identify the epidemiological situation and the animals at risk, giving an indication of whether or not control measures are necessary and what type of control would be suitable for each case.

The inoculation rate is calculated indirectly from the proportion of animals infected, obtained by a serological survey. In *B.bovis* infections, if the inoculation rate is lower than 0.0005 the organism might disappear from the environment if the tick population is under artificial control and there is no need for immunisation. In these situations the disease will tend to be eradicated and an accidental reintroduction would result in outbreaks with great losses.

On the other hand, if the inoculation rate lies between 0.005 and 0.0005 only 25 to 40% of the animals will receive infection between 9 months and 4 years of age and immunisation is necessary to prevent clinical cases.

Inoculation rates higher than 0.005 imply that more than 75% of the calves become infected before 9 months of age and the rest of the animals will be infected before they reach 2 years of age. In that situation, clinical cases are not usually observed and there is no need for control measures. According to Lawrence and de Vos (1990), however, choosing not to carry out active control fails to keep disease at a minimal level under the following conditions, where:

- due to ecological factors, the tick infestation is too low to maintain a critical level for stability
- the tick infestation is too high on susceptible breeds of cattle and causes unacceptable losses
- the tick population varies from year to year due to climatic variations
- cattle are moved to and from areas with different tick populations

Under these situations the use of control programmes should be implemented in order to prevent the occurrence of clinical disease.

Essentially, control measures rely on chemotherapy, tick control and immunisation.

A wide variety of drugs has been used for treatment of acute cases of bovine babesiosis; these include acridine derivatives (acriflavine hydrochloride), quinoline derivatives (quinuronium sulphate), diamidine derivatives (diminazene diaceturate), carbanilide (imidocarb dipropionate) and tetracyclines. The aim of the treatment is to promote clinical recovery allowing some organisms to persist in order to ensure immunity.

Anti-*Babesia* drugs can also be used, at sub-therapeutic doses, prior to or simultaneously with infection to prevent clinical disease (chemoprophylaxis). Due to its slow degradation in the body of the animal, imidocarb is the most suitable drug for use in chemoprophylaxis, which is usually associated with immunisation programmes (Kuttler, 1988), although care

must be taken to correctly time application of drug and subsequent vaccination. However, recent restrictions have been placed on the marketing of imidocarb due to toxic residues in treated animals. Long-acting tetracycline used simultaneously with live *B.bovis* and *B.bigemina* vaccines has been shown to reduce parasitaemia and red blood cell destruction without inhibiting the development of immunity in the vaccinated animals (Pipano et al, 1987; Marcovics et al, 1991). The chemotherapy of babesiosis has been reviewed by Kuttler (1988).

Tick control is usually achieved by the use of chemical acaricides and, due to the influence of climatic factors on the development of ticks, a comprehensive ecological study of each situation is useful to establish any variation in subsequent recommendations for tick control strategies that might be necessary. The various methods currently used for the control of *Boophilus* ticks have been reviewed by Smith and Kakoma (1989) and Nari (1990).

Tick control may be implemented at two levels, eradication or strategic control. Eradication programmes require the provision of effective barriers between tick-free and tick-infested areas and may only be considered appropriate in marginal areas where survival of ticks is tenuous. Eradication of *Boophilus* ticks in the mainland USA, which was completed after a 4-decade period from its initiation in 1906, is the only case of a successful eradication programme (Ristic & Montenegro-James, 1988). In endemic areas, eradication would be extremely demanding in terms of costs and continuous supervision over a long period. Examples of that are given by attempts to eradicate *Boophilus* sp in Mexico (Trapaga, 1989) and in Argentina (Signorini & Mattos, 1989). A more rational action would be based on reduction of the tick population to a level at which transmission of disease is still maintained with perpetuation of a state of enzootic stability. Strategic control involves the application of acaricides at predetermined times during the year when tick infestation exceeds a specific level (Johnston et al, 1981). However, as the transmission rate of *B.bovis* is very low, requiring relatively high numbers of ticks to ensure enzootic stability, the strategic control of ticks may result in a need for concomitant immunisation programmes when *B.bovis* infections are involved.

Immunisation of the animals at risk is the most appropriate method for controlling the disease, since it allows tick control to be directed towards prevention of tick worry. Immunisation programmes are generally implemented in situations where a rigid form of tick control is obligatory, i.e. when infestations by *Boophilus* ticks reach unacceptable levels on susceptible breeds of cattle and represent a limiting factor to beef and milk production. Ideally, immunisation should be achieved by the use of a vaccine able to: *i*) prevent clinical disease under field conditions; *ii*) protect against all strains of the parasite; *iii*) induce long-term protection; *iv*) have no contaminating antigens or infection; *v*) be available in large

quantities at an acceptable cost; *vi*) retain potency during storage and transportation; and *vii*) be safe and easy to administer (Mahoney, 1981; de Vos et al, 1987).

A procedure for induction of protective immunity by inoculation of blood from carriers into susceptible animals and their subsequent treatment, known as premunization, is the oldest method of immunisation against *Babesia* parasites (Pound, 1897). However this method presents serious limitations such as the risk of dissemination of other diseases, the lack of knowledge of the number of parasites being inoculated and consequently the possible induction of severe acute disease with a need for treatment. Furthermore, inoculation of blood may cause deaths from haemolytic disease in calves as a result of induction of antibodies against red blood cell stroma antigens in their mothers (Dimmock & Bell, 1970).

Vaccines against *Babesia* can be classified into two categories: living and non-living vaccines.

Living vaccines were developed in Australia by altering the virulence of the organism through serial passages of infected blood in splenectomised calves (Callow & Mellors, 1966). Vaccines of this type have been successfully used in Australia for the past 20 years and this technique for attenuation has been applied for production of vaccines in South Africa, and in many countries in South America (Taylor, 1989). Vaccine strains of *B.bovis* are passaged, during the acute phase of infection, from a minimum of 20 to a maximum of 30 times in splenectomised calves. Attenuation of *B.bigemina* is achieved by slow serial passages in intact calves which are splenectomised during the chronic phase of infection to produce relapse parasitaemias (Taylor, 1989). In endemic areas where the two infections are present a bivalent vaccine is used. These living attenuated vaccines can be stored and distributed refrigerated (4^{o}C) and have the advantage of being used at a standard infective dose. However the viability of the parasite is limited to a maximum of 10 days, resulting in a very short "shelf life" of the vaccine; another disadvantage of chilled vaccines is that the risk of transmitting other pathogenic organisms still remains. To overcome these limitations, vaccines frozen in liquid nitrogen have been used allowing tests to be carried out for infectivity, immunogenicity and freedom from contaminating infections before distribution (Jorgensen et al, 1989). The major limitation of frozen vaccines, however, is the high cost of their storage and distribution.

The use of the *in vitro* culture systems for provision of cultured attenuated parasites has overcome the problems of foreign disease agents. A recent modification of the MASP culture system for *B.bigemina* has been reported which resulted in a culture suspension system that yielded three times more parasites than the original MASP system; this technique has been

used for vaccine production in Australia (Jorgensen et al, 1992). Nevertheless, increasing the scale of *in vitro* culture systems might be a limitation if the demand for vaccine is high.

Irradiation of *Babesia* parasites has also been attempted for the development of vaccines (Irvin et al, 1979; Wright et al, 1982). The principle is to use an intermediate dose of irradiation that kills most of the parasites and, hopefully, modifies the virulence of the survivors. However, despite the evidence of some degree of homologous and heterologous strain protection with only mild reactions after heterologous challenge, irradiated vaccines have not been developed further.

Non-living vaccines include parasite extracts, supernatant from cultures (exoantigens) and synthetic vaccines developed by recombinant DNA technology.

Crude parasite extracts have been shown to confer strong immunity (Mahoney & Goodger, 1981; Mahoney et al, 1981a), but reduction of immunogenicity occurred as the preparations were purified. Nevertheless, great efforts have been made in the identification and purification of candidate antigens from crude parasite extracts by the use of monoclonal antibody and affinity chromatography technologies. The identification of target antigens is reviewed in section 2.3.3.

Based on findings that circulating antigens in the plasma from animals with acute *Babesia* infections provide a degree of protection in susceptible animals (Sibinovic et al, 1967; Mahoney & Goodger, 1972), supernatants from cultures of *B.bovis* and *B.bigemina* have been tested for use as non-living vaccines (Smith et al, 1979; Montenegro-James et al, 1985). These exoantigens, which are thought to be composed of three components of which one or more originated from the merozoite surface coat (Smith et al, 1981), have been used in field vaccination trials in some Latin American countries providing satisfactory protection. However, protection conferred by *B.bovis* culture supernatant has been shown to be only partial after challenge with heterologous strains and to be substantially lower than the protection conferred by live parasites (Timms et al, 1983; 1984). The use of this type of vaccine would offer several advantages over the currently used attenuated live vaccines. These include the possibility of lyophilization which would facilitate storage and transportation, and elimination of occurrence of neonatal haemolytic disease, since culture supernatant is believed to be virtually free of red blood cell stromal antigens.

With the advent of recombinant DNA technology, great efforts have been made in the development of a new generation of babesial vaccines based on the cloning of relevant genes which express particular parasite proteins. These include studies on parasite surface proteins (Reduker et al, 1989; McElwain et al, 1991; Suarez et al, 1991), and the sub-fractionation of protective fractions of native proteins with the aim of identifying protective epitopes

(Commins et al, 1985; Goodger et al, 1985b). The first trial of a vaccine for *B.bovis* derived from recombinant DNA, however, showed that significant protection against challenge was not produced (Timms & Barry, 1988). Despite that unsuccessful attempt, many workers believe that the ideal babesiosis vaccine will require DNA technology with the most recent approach being the combination of recombinant strain-conserved protective proteins in a "cocktail" which may confer complete protection to susceptible cattle (Gale et al, 1992; McElwain et al, 1992).

2.3 IMMUNOLOGY

Immune responses in babesiosis are complex and involve both humoral and cell-mediated components (de Vos et al, 1987, Zwart & Brocklesby, 1979).

2.3.1 Humoral immune response

The first evidence that antibodies play a protective role in *Babesia* infections was given by the passive transfer of maternal immunity to calves by the antibodies present in colostrum (Hall, 1960; Hall et al, 1968). However, attempts to correlate antibody levels with immune status have failed, suggesting that not all antibodies are protective (Mahoney, 1964; Callow et al, 1974).

On the other hand, further evidence of a protective role for antibodies was given by Mahoney, who showed that passive transfer of immune serum to susceptible splenectomised calves resulted in protection against *B.bovis* (Mahoney, 1967a). Later, it was found that protection was due to IgG, with the target antigens appearing to be located on the surfaces of infected red blood cells and parasites, and that the antibodies acted as opsonins with the organisms being killed by phagocytes (Mahoney et al, 1979). However, cross-protection between antibodies directed against heterologous strains was not observed and it was suggested that strain-specific immunity and variant-specific serological activity during chronic infections result from different antigen-antibody reactions (Mahoney et al, 1979).

In vitro studies of rodent babesias have shown that antibodies suppress parasite growth by inhibition of parasite entry into erythrocytes (Bautista & Kreier, 1979). Opsonic activity correlated with the protective function of serum has been demonstrated by the passive transfer of immunity in *B.rodhaini* (Rogers, 1974). Immune serum also increased *in vitro* phagocytosis of *B.gibsoni* by blood monocytes, and in *B.microti* cultures immune serum acted together with macrophages to inhibit growth (Bautista & Kreier, 1980).

Furthermore, it has been reported that free merozoites are particularly susceptible to attack by antibodies (Thoongsuwan & Cox, 1973; Abdalla et al, 1978; Smith et al, 1981). Ristic and Levy suggested that antigens located on the surface coat of merozoites are the major antigenic determinants associated with protective immunity to *Babesia*, and that the antibodies to these antigens are involved in disruption and lysis of the organism (Ristic & Levy, 1981).

In contrast, it has been reported that protective immunity is inversely related to antibody titre when using defined protective antigens (Wright et al, 1983a; Goodger et al, 1986).

In *Babesia* infections antibodies are produced, not only against a broad spectrum of parasite antigens, both protective and non-protective, but also against host antigens as the result of an inflammatory response (Goodger et al, 1985a). According to Goodger and co-workers, most of the antibodies detected by serological assays are non-protective (Goodger et al, 1985a).

Non-specific factors, such as fibronectin, conglutinin and complement (C3) are involved in immunity against babesiosis with low titres of protective antibodies being sufficient for protection (Goodger, 1989). Complement also seems to play an important role in the invasion of rat erythrocytes by *B.rodhaini* (Jack & Ward, 1980). However serum complement does not appear to interfere with *B.bovis* invasion and does not facilitate invasion of bovine erythrocytes *in vitro* (Levy et al, 1986).

In general, antibody levels rise to an early peak and decline during the chronic stage of infection (Smith et al, 1978). In endemic areas, calves receive passive protection from maternal colostral antibodies until about three months of age, by which time they have usually become infected with the peak of antibodies occurring at 4 to 5 months of age (James et al, 1985).

The kinetics of antibody levels in *B.bigemina* infections have been studied using an enzyme immunoassay by O'Donoghue and co-workers, who reported the appearance of IgG at 7 days after infection, with peak titres from 12 days and persistence of a plateau for 7 weeks (O'Donoghue et al, 1985). IgM antibodies appeared 7 days after infection, peaked at 12 days, persisted at maximum levels until day 22 and then declined to low levels by 28 days after infection.

2.3.1.1 Antigenic heterogeneity of parasite populations

The first evidence of antigenic diversity of *Babesia* parasites was given by Curnow, who used a parasitized erythrocyte agglutination test to demonstrate antigenic differences between seven Australian isolates of *B.bovis*, indicating the presence of multiple strain-specific parasite antigens (Curnow, 1968; 1973). The latter study demonstrated the emergence of different variants of the parasite during the chronic phase of the infection, which reverted to the basic antigenic type after transmission by the tick vector. The variant antigens appeared at intervals of approximately three weeks resulting in recrudescence of parasitaemia. *In vivo* evidence of strain differences in *B.bovis* and *B.bigemina* infections was given by studies on cross-immunity in which animals recovered from infection were more resistant to homologous challenge than to challenge with a heterologous strain (Callow, 1964; Uilenberg, 1970).

Studies on the protein composition of different parasite populations by 2-dimensional gel electrophoresis of biosynthetic labelling of infected erythrocytes have demonstrated antigenic

differences both between strains and between virulent and avirulent forms of the same strain (Kahl et al, 1982; 1983). These studies revealed that some of the antigens which were present or absent in different populations differed in their amino acid composition, and there was evidence that protein differences may be correlated with virulence.

Differences between populations of *B.bovis* have also been demonstrated by genetic analysis. A number of genes expressed in attenuated lines, but not in virulent lines, have been identified and their use as probes showed that strain diversity and the selection of genetically distinct subpopulations of parasites occurred during passage either through cattle or *in vitro* culture (Cowman et al, 1984; Carson et al, 1990). Further evidence of genetic and antigenic polymorphism among populations of *B.bovis* was given by the finding that cloned populations originating from the attenuated Ka strain exhibited distinct antigenic differences, with one clone being significantly more virulent than the others, and none of the cloned populations being transmissible by ticks (Gill et al, 1987a). Although that study indicates that there is considerable heterogeneity within parasite strains, it suggested that a major component of the immunity was not parasite strain-specific, since the animals infected with cloned parasites were immune to challenge with a different strain.

Further investigations on the virulence of cloned lines derived from the avirulent vaccinal strain (Ka) have been reported by Timms and co-workers, who found variability in virulence, from some clones which were completely avirulent to others which were highly virulent (Timms et al, 1990). In that study, none of the cloned lines were transmissible by the tick vector, neither alone nor as a combination, which is in direct contrast to the parent strain being readily tick transmissible. The authors suggested the possibility that a gene product required for tick transmission, perhaps an enzyme necessary for penetration of the gut epithelial cells of the tick, had been lost in these cloned populations. They further suggested that field strains of *B.bovis* contain both tick-transmissible and non-transmissible subpopulations with the transmissible parasites being capable of supporting the transmission of the non-transmissible parasites by provision of a factor required for transmission.

The reversion to virulence of an attenuated strain of *B.bovis* has been demonstrated after mechanical passage through intact cattle or biological passage through ticks (Callow et al, 1979; Kahl et al, 1983). Timms and co-workers also reported reversion to virulence of one of their clones after passage through an intact calf and suggested that the genes related to virulence must still be present, but not expressed, in both the Ka and the cloned avirulent strains (Timms et al, 1990).

The diversity of *B.bovis* parasites may have practical implications for vaccination programmes (Dalrymple, 1992). In the late 1980s significant problems were observed in Australia with the attenuated strain (Ka) of *B.bovis* used as a live vaccine. These problems

were related to an increased number of vaccinated animals showing severe reactions after challenge. Dalrymple suggested that these problems could be attributed either to a change in the composition of the vaccine (both phenotypic and genetic) or to the selection of some resistant strains in the natural population, or to a combination of both factors. The selection of parasite subpopulations that could evade the host immune system, as a result of the intensive use of one vaccine line of parasite, has not yet been demonstrated in *B.bovis*. However, if it is found to be the case, strain diversity of *Babesia* parasites may have even more significant implications for the success of the development of recombinant vaccines, which will inevitably be based on the use of a small number of parasite components.

A more comprehensive analysis of subpopulations of *B.bovis* has been reported recently by Dalrymple and co-workers, who used a recombinant DNA probe specific for a tandemly repeated gene of this parasite to analyse a number of samples from different isolates of *B.bovis* (Dalrymple et al, 1992). That study revealed that five out of seven samples originally derived from single clinical cases contained more than one distinct subpopulation of parasites, while only one population was identified in samples from the Ka line used as the live attenuated vaccine strain in Australia from 1979 until 1990. On the other hand, the Ta line, which replaced the Ka line as an attenuated vaccine strain, was shown to contain two genetically distinct subpopulations of parasites. One of the *B.bovis* lines analysed by Dalrymple and co-workers, the Lismore (LP) strain which originated from the Lismore isolate used in the present study (Timms, personal communication), was shown to comprise a homogeneous population. Furthermore, the same subpopulation was detected after the Lismore (LP) line was passaged through ticks, although the parasites appeared to be more virulent. Thus, no correlation between the increase in virulence and selection of a major distinct subpopulation of parasites was observed. The results obtained from the genetic characterisation of the different isolates of *B.bovis* carried out by Dalrymple and co-workers indicated occurrence of changes in the composition of parasite populations after passage through splenectomised calves and in tissue culture (Dalrymple et al, 1992).

2.3.2 Cellular immune response

Lymphocytes (B and T) and natural killer cells (NK) are involved in the immune mechanisms activated in response to *Babesia* infections (de Vos et al, 1987). Elimination of *B.microti* in mice has been reported to be thymus-dependent (Clark & Allison, 1974; Ruebush et al, 1980a). Further evidence of a role for T-cells in the development of resistance to *B.rodhaini* and *B.microti* was given by the transfer of immunity with lymphoid cells from infected mice to either susceptible or irradiated mice (Roberts, 1968; Ruebush & Hanson, 1980b). Lymphocyte transformation has also been reported in *B.bovis* infection with a more

pronounced effect produced by inoculation of soluble supernatant from cultures than with live vaccine (Timms et al, 1984). The assay showed that the cell-mediated response was short-lived, with a relatively low stimulation index being recorded 10 to 18 days after vaccination with live parasites. In contrast, positive stimulation was observed throughout a 6-month period with maximum values being observed after 18 weeks, following vaccination with soluble exoantigens.

Machado and co-workers, evaluating the cell-mediated response to *B.bovis* infection using a leucocyte migration inhibition test, reported significant inhibition of migration as early as 6 days after infection, and continuously high levels of inhibition throughout the 26-day observation period (Machado et al, 1985).

The evidence for an antibody-dependent cell-mediated cytotoxicity (ADCC) mechanism being involved in the resolution of *B.bovis* infections was given by studies of Goff and co-workers, who demonstrated enhancement of ADCC in mononuclear effector cells isolated from the peripheral blood of infected cattle (Goff et al, 1984a,b). The levels of activity increased during the parasitaemic crisis (between 5 to 13 days after infection) and returned to normal levels with the resolution of parasitaemia (19 days after infection).

The most comprehensive studies on the cell-mediated response to *B.bovis* infections have been described recently by Brown and co-workers in a series of reports which started with the examination of peripheral blood mononuclear cells (PBMC) from cattle experimentally inoculated with tick-derived or cultured parasites tested against different parasite antigenic fractions (Brown et al, 1991). Results from that study demonstrated that infection with *B.bovis* elicited a strong cell mediated response. However, animals inoculated with tick-derived parasites preferentially responded to an antigenic fraction from parasite membranes, whereas inoculation of cultured merozoites elicited a vigorous response to a parasite membrane-enriched fraction, a soluble merozoite fraction, and soluble supernatant antigens. Furthermore, it was found that the antigens most immunogenic for T cells are parasite membrane-enriched fractions and that monocytes appear to be required as antigen-presenting cells in the *Babesia*-specific response. There was also indication that *B.bovis* and *B.bigemina* share T-cell epitopes, since *B.bovis*-immune PBMC responded to *B.bigemina* parasites.

T cells are believed to be central to both humoral and cell-mediated responses in *Babesia* infections, and to be critical for activating macrophages by the elaboration of cytokines (Brown et al, 1993). Nevertheless, studies on the cellular immune response to a protective antigen, in both its native and recombinant forms, failed to induce interferon gamma production by cells from vaccinated cattle when stimulated by *B.bovis* antigens *in vitro*, suggesting that immunity is correlated most strongly with humoral rather than cell-mediated responses (Orinda et al, 1992a).

Further studies by Brown and colleagues on the role of T helper cells (Th) in *B.bovis* infections demonstrated preferential stimulation of $CD4^+$ T cells by unfractionated cytosolic and merozoite membrane antigens (Brown & Logan, 1992). A panel of 11 Th clones derived from several *Babesia*-specific T cell lines, which had demonstrated functional heterogeneity, were further characterised on the basis of the patterns of proliferative responses against merozoite fractions from different geographical isolates of *B.bovis* (Mexico, Texas and Australia) and *B.bigemina* (Mexico) (Brown et al, 1992). In that study, at least five distinct epitopes of *B.bovis* merozoite antigens were distinguished and classified according to their differential Th cell responses. One group of four clones responded to a parasite membrane epitope present in all *B.bovis* isolates, but not in *B.bigemina*; the remaining clones responded to epitopes present in both parasite membrane and soluble fractions from merozoites, and included one clone which recognised an antigenic epitope shared by all *B.bovis* stocks and *B.bigemina*, and five clones which recognised an antigen present only in the Texan and Mexican isolates, but not in the Australian *B.bovis*. These findings allowed the identification of two Th cell epitopes on a merozoite protein (Bb-1) which appeared to be associated with induction of protection, with further studies being directed to support its incorporation into a recombinant vaccine (Brown et al, 1992).

2.3.2.1 The role of the spleen

In most mammalian hosts the spleen plays an important role in the development of immunity against *Babesia* spp and in the suppression of infection in the chronic stage of infection (Phillips, 1970; Mahoney, 1977). Splenectomy results in increased susceptibility and also allows the establishment of infection in hosts that are naturally immune to infection, including humans. In cattle with latent *B.bovis* infection, splenectomy rarely results in a patent relapse parasitaemia, whereas in *B.bigemina* infections splenectomy usually precipitates nonfatal parasitaemias (Carson & Phillips, 1981).

The mechanisms by which the spleen affects the infection appear to be related to three functions: i) removal or sequestration of parasitized erythrocytes from blood as a result of "pitting", as described for malaria parasites (Schnitzer et al, 1972), which results in reduction of parasite numbers before the development of a specific immune response; ii) erythrophagocytosis; and iii) production of specific antibodies, T-cells, NK cells and soluble nonantibody mediators (Carson & Phillips, 1981; de Vos et al, 1987).

Spleen cells from immune rats transferred to susceptible rats confer protection against *B.rodhaini* (Roberts, 1968). Similarly, immunity to *B.microti* can be transferred more efficiently to susceptible mice with spleen cells from immune animals than with cells from

other lymphoid organs (Allison et al, 1979). Although neither splenic microcirculation nor mature T-cells appeared to be essential for immunity against *B.microti*, protection is more rapidly established in intact than in splenectomised mice (Allison et al, 1979).

Phagocytosis of infected erythrocytes occurs in the spleen and the splenic microcirculation appears to facilitate this function by a process of "sequestration" of infected cells from the circulating blood (de Vos et al, 1987). It has been suggested that phagocytosis occurs after immune complexes composed of soluble parasite antigen, antibody, complement and immunoconglutinin have attached to infected erythrocytes (Thoongsuwan et al, 1978). Phagocytosis of *B.rodhaini* infected erythrocytes and of free parasites has been demonstrated in an *in vitro* model using mouse macrophages (Parrodi et al, 1991). More recently, the same *in vitro* model was adapted for *B.bovis* infected cells in an attempt to identify the role of protective antibodies as opsonins in *B.bovis* infections (Jacobson et al, 1993). The study showed that *B.bovis* immune sera failed to promote phagocytosis, whereas antibodies raised against protective fractions obtained by dextran sulphate precipitation (Goodger et al, 1990) consistently induced phagocytosis. These findings suggested that phagocytosis induced by opsonized particles may play an important role in the mechanism of protection. Furthermore, NK activity increases in infected animals prior to elimination of parasites (Irvin et al, 1981).

Although it has been demonstrated that synthesis of antibodies occurs in extra-splenic sites (Phillips, 1970), complement fixing antibodies appeared 5 to 6 days later in splenectomised sheep than in intact animals following infection with *B.ovis*, suggesting that splenectomy may reduce protective antibody production (Zwart & Brocklesby, 1979).

2.3.3 Target antigens

The definition of specific parasite antigens responsible for induction of protective immunity has been the general approach towards the development of improved methods of immunisation against *Babesia* spp. Three contrasting lines of research have been adopted to achieve this. The first approach has focused on the identification of immunodominant parasite surface antigens using polyclonal and monoclonal antibodies in techniques such as immunoblotting and immunoprecipitation of radiolabelled proteins, and the subsequent evaluation of these antigens as protective immunogens in vaccination trails (McElwain et al, 1992). The second approach has been based on indications that immunodominant antigens do not induce a protective immune response and therefore it is necessary to evaluate the protection conferred by parasite fractions in vaccination trials before further characterisation

takes place (Goodger et al, 1992b). A third approach has focused on selection of antigens based on biological and pathophysiological parameters (Commins et al, 1985).

2.3.3.1 *B.bovis* antigens

Immunoprecipitation of ^{35}S-methionine radiolabelled *in vitro* cultured parasites by polyclonal sera allowed the identification of eight proteins with molecular weights from 14.5 to 200 kDa, with one of them (a 42 kDa protein) shown to be *B.bovis* strain-common and species-specific (McElwain et al, 1988).

Indirect fluorescence of live *B.bovis* merozoites and immunoprecipitation of ^{125}I labelled merozoite surface proteins by polyclonal and monoclonal antibodies resulted in identification of four proteins with molecular weights of 37, 42, 60 and 85 kDa (Goff et al, 1988). The 37 and 42 kDa proteins were shown to be distributed over the entire surface of merozoites, whereas the 60 kDa protein appeared to be punctate, polar and present in only about 5% of the total viable merozoites; the exact location of the 85 kDa protein was not determined.

Later, a panel of ten monoclonal antibodies generated against five *B.bovis* merozoite surface proteins (molecular weights of 16, 42, 44, 60 and 225 kDa) was used to screen a genomic DNA library resulting in the identification of two recombinant clones that encoded a protein recognised by a monoclonal antibody for an epitope on the native 44 kDa protein (Reduker et al, 1989). The recombinant form of the 44 kDa protein was used to immunise cattle. Results from the immunisation trial showed that a specific immune response was generated, however the level of antibodies among vaccinated animals was highly variable (Reduker et al, 1989).

Further studies on immunoprecipitation of merozoite surface proteins of a cloned and pathogenic isolate of *B.bovis* by polyclonal sera from protected cattle identified eight proteins (Hines et al, 1989). Among them, a 42 kDa transmembrane protein was consistently recognised at the highest dilution of immune sera (1: 16,000) and was identified as a potential candidate for further studies.

A *B.bovis* antigen with a molecular weight of 44 kDa was also identified by immunoadsorption with the corresponding monoclonal antibody (15B1) and was shown to induce protective homologous immunity in splenectomised calves (Wright et al, 1983b).

Analysis of a saline eluate from sucrose washed bovine erythrocytes infected with *B.bovis* by Western blotting with rabbit antiserum raised against the eluate allowed the identification of four antigens with molecular weights of 120, 190, 280 and 380 kDa (Goodger & Wright, 1983). Immunofluorescent staining showed that the eluate contained proteins from

membranes of non-infected erythrocytes and, in addition, proteins which were located not only in the parasite but also in the internal matrix of infected erythrocytes.

In view of the role of esterase in the coagulation and kinin systems of the host during *Babesia* infections, investigations have been directed towards determining its role in the protective immune response (Wright et al, 1983a). Thus an esterase was isolated from a crude extract of *B.bovis* by affinity chromatography and used to immunise susceptible cattle. The results from that study showed that the vaccinated animals underwent severe reactions, after challenge with either homologous or heterologous strains of the parasite, although a specific antibody response was demonstrated by immunofluorescence and immunoblotting.

Protective immunity against heterologous challenge was conferred when two *B.bovis* fractions, which were obtained from a lysate of infected erythrocytes by a gel filtration procedure using Sephadex G200, were used to immunise susceptible cattle (Goodger et al, 1984). In the same study, when these fractions were analysed by Western blotting against the corresponding antiserum, one fraction was shown to produce six reactive bands with molecular weights between 27 and 300 kDa, whereas the second fraction produced four bands with molecular weights of 70, 60, 44 and 27 kDa. Erythrocyte iso-antigens were also detected by reactions of normal plasma against antigens present in both fractions, suggesting that further fractionation was required for purification of protective parasite antigens. Further studies carried out by Goodger and co-workers showed that the second fraction, which contained dominant immunodiffusion antigens, failed to induce protection against *B.bovis* in susceptible calves (Goodger et al, 1986).

A low molecular weight antigen (29 kDa), which was purified from a lysate of *B.bovis* infected erythrocytes, was excised from acrylamide gels and used to immunise susceptible adult cattle (Wright et al, 1985). Challenge with the homologous strain of *B.bovis* showed that immunised animals were partially protected.

It has also been reported that antigenic preparations of *B.bovis* contain dominant components that react in Western blotting not only with antisera to *B.bovis* parasites but also with sera from naive calves recovering from an acute inflammatory reaction, indicating that some antigens originate from the host rather than the parasite (Goodger et al, 1985a).

Two *B.bovis* proteinases, with molecular weights of 22 and 70 kDa, have also been identified, and the latter was used in a vaccination trial where the immunised cattle were shown to be protected after being challenged with the virulent strain from which the proteinases had been isolated (Commins et al, 1985).

A heparin binding fraction of a supernatant obtained by ultracentrifugation of a lysate of *B.bovis* infected erythrocytes, was used in a vaccination trial in which the immunised

splenectomised calves were shown to be protected after challenge with the homologous strain of the parasite (Goodger et al, 1987a). When analysed by Western blotting, this fraction was found to contain immunodominant bands of 180 kDa, a doublet of 140 kDa, and a weak band of 135 kDa (Goodger et al, 1987a).

Gill and co-workers used recombinant DNA techniques to produce a cDNA clone coding for a portion of a high molecular weight antigen of *B.bovis* (Gill et al, 1987a). In that study, a *B.bovis* cDNA library was screened with hyperimmune bovine serum and an antigen-positive clone, which encoded a 5-10 kDa polypeptide of a 220 kDa immunodominant *B.bovis* native protein localised at the apex of the parasite, was selected. Later, its recombinant form fused to β-galactosidase was used to immunise susceptible cattle in a vaccination trial; however protection was not observed when the immunised animals were challenged with a virulent heterologous strain of *B.bovis* (Timms & Barry, 1988).

More recently, a chloroform extract from *B.bovis*-infected erythrocytes was used to vaccinate susceptible cattle, which were challenged with a virulent heterologous strain, resulting in delayed and decreased parasitaemias when compared to the control unvaccinated group of cattle (Goodger et al, 1990). In the same study, biochemical analysis of ^{14}C-labelled infected erythrocytes suggested that the immune response was elicited by lipids of babesial origin which comprised four bands of 12 to 18 kDa in Western blotting analysis.

Another lipid-enriched fraction of *B.bovis* was isolated from infected erythrocytes by hexane extraction; it showed antigenic and immunogenic activity in ELISA using either bovine antiserum to *B.bovis* and *B.bigemina* from natural infections or rabbit antiserum to the hexane extract (Orinda et al, 1992b). Use of IFAT demonstrated that this fraction was associated with the parasite and infected erythrocytes, and was not present in non-infected erythrocytes; cross-reactions with *B.bigemina* antisera were observed, suggesting that serological cross-reactivity in *Babesia* species might be due to lipid or lipid-associated antigens.

A multifractionation procedure for *B.bovis* infected erythrocytes, involving selective lysis, sonication, ultracentrifugation, gel filtration and preparative agarose gel electrophoresis, resulted in the selection of a fraction, designated "β", which elicited protection of susceptible cattle after challenge with a heterologous strain of the parasite (Goodger et al, 1985b). From several monoclonal antibodies produced against this fraction, one designated W11C5 was selected and used to extract by affinity chromatography the corresponding antigen from *B.bovis* infected erythrocytes. The W11C5 monoclonal antibody was shown to react against infected erythrocytes by IFAT and to give a large number of closely positioned reactive bands in Western blots (Goodger et al, 1992b). The extracted antigen was then used in a vaccination trial to test its efficacy in protecting splenectomised calves against homologous challenge; the

vaccinated group showed significantly lower parasitaemias in comparison to the control group (Goodger et al, 1992b). These authors suggested that the production of the *B.bovis* W11C5 antigen by the use of recombinant DNA techniques would allow the evaluation of its suitability as a commercial vaccine against *B.bovis* infections.

The use of antigens produced by recombinant DNA technology in vaccination against *B.bovis* has recently been reviewed by Gale and co-workers, who reported that a recombinant W11C5 fusion protein has been produced and its gene has been sequenced (Gale et al, 1992). They also reported that the recombinant W11C5 protein induced protection in susceptible cattle in a vaccination trial in which a heterologous strain of *B.bovis* was used for challenge. In that trial the vaccinated animals developed significantly lower parasitaemias and exhibited increased survival rates when compared to the control group.

Another antigen (12D3), which had originally been isolated from a protamine sulphate precipitated fraction (Wright et al, 1983a), has also been targeted as a protective antigen, and its recombinant form induced significant reduction in parasitaemia in immunised animals upon *B.bovis* challenge (Gale et al, 1992). DNA hybridisation studies have revealed that the 12D3 gene is conserved in a range of different species of *Babesia*, with homologues of the gene being detected in *B.bigemina*, *B.canis*, *B.bovis* and *B.divergens*. Gale and co-workers also reported that, in a field immunisation trial, an antigen 'cocktail' of W11C5 and 12D3 induced protection in 90% of the animals, and that a third recombinant antigen (designated T21B4), which was originally identified as a protease protective antigen (Commins et al, 1985), has been included in an attempt to further improve vaccine efficacy (Gale et al, 1992). It was also reported that vaccination trials are currently under way to test the efficacy of the trivalent vaccine under a range of field challenge conditions.

2.3.3.2 *B.bigemina* antigens

The general approach towards identification of *B.bigemina* antigens has been through the use of monoclonal antibodies developed against merozoites to screen for surface reactivity. Immunoprecipitation of ^{35}S methionine-labelled parasite proteins by monoclonal antibodies identified five merozoite proteins with molecular weights of 72, 58, 55, and 45 kDa (McElwain et al, 1987).

In another report, surface reactive monoclonal antibodies identified parasite components with molecular weights ranging from 68 to 36 kDa, including the same 58 kDa identified by McElwain and co-workers (1987), which were conserved in at least six geographically different *B.bigemina* isolates (Figueroa et al, 1990b).

In vitro radiolabelling and immunoprecipitation studies indicate that post-translational modification of *B.bigemina* proteins is common, and that at least ten parasite proteins are post-translationally modified by incorporation of glucosamine, whereas seven are modified by incorporation of myristic acid (McElwain et al, 1991). The p58 protein was not modified by the incorporation of either glucosamine or myristic acid and appeared to be conserved among *B.bigemina* isolates from Mexico, Puerto Rico, St. Croix and Kenya. Furthermore, it was interpreted that calves immunised with each of three immunoaffinity purified proteins (gp45, gp55 and p58) were able to neutralize the infectivity of merozoites as indicated by reductions in the peak parasitaemia after challenge (McElwain et al, 1991).

Two additional monoclonal antibodies have been identified that bind to the membrane of infected erythrocytes in acetone-fixed but not unfixed antigens in IFAT; these monoclonals immnunoprecipitate a ^{35}S-methionine-labelled polypeptide of > 200 kDa (McElwain et al, 1992).

Subsequently, the gene encoding the protein p58 was cloned, expressed, sequenced and characterised (Mishra et al, 1991), and the current approach has been evaluation of the p58 protein, alone or in combination with the high molecular weight membrane protein (> 200 kDa), as an immunogen in vaccination trials, definition of the polypeptides present on the infected erythrocyte surface, and evaluation of the efficacy of expression vectors for presentation of antigens (McElwain et al, 1992).

2.4 BOVINE BABESIOSIS IN BRAZIL: CURRENT SITUATION

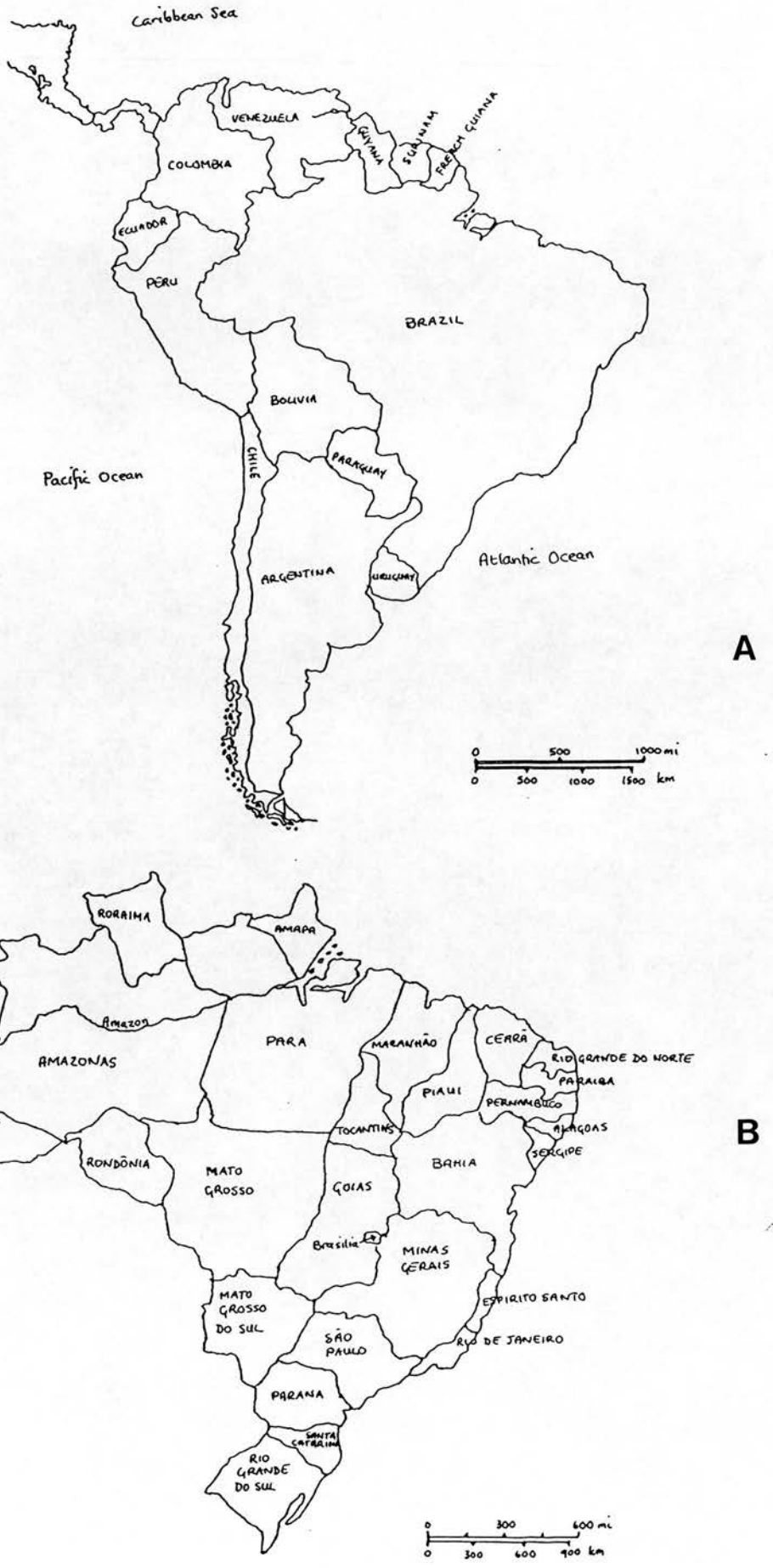
Brazil, officially the Federative Republic of Brazil, with a human population of 151,381,000 (1992 estimation, Encyclopaedia Britannica, 1993) is located in South America, bordered by Venezuela, Guyana, Surinam, and French Guyana to the north; Colombia, Peru, and Bolivia to the west; Paraguay, Argentina, and Uruguay to the south west; and the Atlantic Ocean to the east (see Fig 2.3). It is a federation of 23 states, three territories, and the federal district of Brasilia. The capital is Brasilia, and principal cities include Sao Paulo, Rio de Janeiro, Recife, Belo Horizonte, and Salvador. The largest South American country (3,286,470 sq. miles), Brazil occupies nearly half of the continent and has a varied topography and climate, ranging from tropical in the rain forests of the great Amazon basin in the north to temperate in the highlands of the comparatively heavily populated east and south, which make up two thirds of the country's land mass and contain its chief economic centres. Despite rapid industrialisation beginning in the 1960s, Brazil still depends heavily on agriculture, which accounts for nearly 50% of its exports. It is an important cattle producer; major commercial crops are coffee (of which it is the world's leading producer), cocoa, cotton, sugarcane, citrus fruit, maize, tobacco, bananas, and soybeans. Industrial production is led by motor vehicles, steel, cotton textiles, paper, fertiliser, cement, and machinery. The country's vast mineral wealth includes some of the finest iron resources in the world, as well as quartz, coal, manganese, chromium, industrial diamonds, uranium, and platinum. Petroleum deposits have not been fully developed; petroleum accounts for 26% of imports. Portuguese is the official language and Roman Catholicism the predominant religion (Columbia Encyclopaedia, 1992).

The cattle population of Brazil is around 140 million animals, 50% of which are dairy cattle, and the problem of tick infestation and tick-borne diseases assumes tremendous economic proportions, in terms of lost animal protein and export earnings for Brazil.

According to Rouse (1977), cited by Primo (1992), cattle were brought to the Caribbean in 1493 by the Spanish colonisers and within 40 years the descendants of these animals had been spread throughout the South American countries, mainly by way of Peru, Bolivia, Chile and Paraguay. In Brazil cattle were first introduced in 1534, through Sao Paulo, Pernambuco and Bahia; some of the animals possibly originated from the Cape Verde Islands, a Portuguese colony in the Atlantic Ocean (Primo, 1992). Systematic importations of zebu cattle from India began at the end of the 19th century and continued until 1930, with one further importation in 1952. The first large scale importation of zebu cattle happened in 1906 and included Hissar, Malvi, Mewati and Mysore breeds. Between 1918 and 1921 larger importations took place and up to 1952 over 5,800 zebu cattle had been imported, almost all from India (Maule, 1990).

Fig 2.3 Map of South America (A) and Brazil (B).

Fig 2.3


Caribbean Sea
VENEZUELA
GUYANA
SURINAM
FRENCH GUIANA
COLOMBIA
ECUADOR
PERU
BRAZIL
BOLIVIA
CHILE
PARAGUAY
Pacific Ocean
ARGENTINA
URUGUAY
Atlantic Ocean
A
0 500 1000 mi
0 500 1000 1500 km
RORAIMA
AMAPA
AMAZONAS
PARA
MARANHÃO
CEARÁ
RIO GRANDE DO NORTE
PARAIBA
PIAUI
PERNAMBUCO
ALAGOAS
SERGIPE
ACRE
RONDÔNIA
TOCANTINS
BAHIA
MATO GROSSO
GOIAS
Brasília
MINAS GERAIS
ESPIRITO SANTO
MATO GROSSO DO SUL
SÃO PAULO
RIO DE JANEIRO
PARANA
SANTA CATARINA
RIO GRANDE DO SUL
B
0 300 600 mi
0 300 600 900 km

The three breeds which have contributed most to the Brazilian cattle industry are Guzera, Nelore and Gir, which gradually became adapted to Brazilian conditions.

Nowadays, Nelore is the most numerous zebu breed used for intensive beef production in the upland states of Sao Paulo, Minas Gerais and in central Brazilian regions, while Gir cattle have been developed more for milk production.

With the aim of improving milk production, importation of European breed (*Bos taurus*) began in the 1960s in many countries of South America. These were mainly from the United States and consisted of Holstein-Friesian and Brown Swiss, which were either maintained as pure breeds or crossed with zebu cattle. Large numbers of animals of European breed were imported over the past few decades and this policy is still adopted by some farmers nowadays. However, with the introduction of *Bos taurus* breeds, tick infestation became one of the most serious problems for the Brazilian cattle industry.

The main species of tick infesting cattle, *Boophilus microplus,* was probably introduced into Brazil through cattle imported from Chile which entered the State of Rio Grande do Sul in the 17th century (Thiesen, 1979). Nowadays this tick is distributed all over Brazil causing substantial economic losses; in the State of Rio Grande do Sul for instance, it was estimated in 1974 that total losses of 30 kg per animal up to slaughtering age were due to tick infestations (Vidor, 1975, cited by Beck, 1979).

A survey carried out by the Brazilian government in 1982 showed that commercial sales of drugs against ectoparasites totalled around 45.8 million US dollars, representing 17.2% of all the products marketed for veterinary use (Brazilian Agriculture Ministry, 1983).

Tick-borne diseases of cattle, mainly babesiosis and anaplasmosis, are endemic, causing mortality and disease in livestock and therefore acting as a barrier to the improvement of production and productivity of the herds as well as causing significant economic losses.

In order to increase productivity, most Brazilian farmers have introduced, over the past few decades, *Bos indicus* x *Bos taurus* cattle into their herds. However it has been reported that in Brazil, as in other tropical countries, tick infestation levels increase as the proportion of *Bos taurus* "blood" increases (Gomes et al, 1989; Oliveira & Alencar, 1990). Furthermore, the use of improved pastures has increased, allowing a higher number of animals to be mantained per unit area . As a result, the natural equilibrium of the cattle-tick system is affected. Therefore, management practices relating to tick and tick-borne disease control have became increasingly important, resulting in substantial increases in costs of production.

Several studies on the tick *B.microplus* have been carried out in Brazil since the pioneer work initiated with the foundation of the Oswaldo Cruz Institute, Rio de Janeiro, in 1900; these include studies on the systematic classification (Aragao, 1911; 1917; 1936; Aragao &

Fonseca, 1961) and on resistance of ticks to acaricides (Freitas, 1950; Queiroz & Mello, 1960). However, in the late 1970s, as a result of development of tick resistance to organophosphate acaricides, research on *B.microplus* was directed towards understanding the bio-ecological aspects which affect the interrelationship between cattle and ticks with the aim of proposing strategic programmes to control *B.microplus* ticks (Arteche & Laranja, 1979; Evans, 1979; Souza & Gonzales, 1980; Magalhaes & Lima, 1991).

The duration of each stage of the life cycle of the one host tick *B.microplus* is particularly affected by climatic conditions (temperature and humidity). Under optimum climatic conditions, *B.microplus* shows the following life cycle in Brazil (Arteche, 1975, cited by Beck, 1979; Gonzales et al, 1975):

Stage of the tick life cycle	Minimum duration (days)	Maximum duration (days)
a) Non-parasitic stage:		
1. from detached engorged female to initiation of oviposition	2	23
2. oviposition	14	44
3. pre-eclosion	14	202
4. new-born larva to attached larva	6	184
subtotal	36	453
b) Parasitic stage:		
from attached larva to engorged female	18	48
total	54	501

A study on the effect of climatic conditions on *B.microplus* infestation of 1-2 year old 'Mantiqueira' type heifers which had been dipped with acaricide 35 days before the tick counting, was carried out in a district of Sao Paulo State and resulted in average counts of 22, 74, 93 and 10 semi-engorged females per animal, in spring, summer, autumn and winter, respectively (Guaragna et al, 1988).

A more comprehensive study was carried out in a district (Pedro Leopoldo) of Minas Gerais State, in which several climatic parameters were analysed in relation to the development of

the *B.microplus* life cycle under field conditions, throughout a four-year period between November 1983 and November 1987 (Magalhaes, 1989). Pedro Leopoldo is located at 882 m altitude, and has a dry winter with an average temperature of 16°C, and a rainy summer with an average temperature of 25°C (Antunes, 1986). For a study of the non-parasitic stage of the ticks, engorged females were distributed on a defined area of pasture at 14-day intervals, and the egg incubation period and survival of larvae were registered. To study the parasitic stages, tick-free cattle were introduced into naturally infested pastures and the number of semi-engorged female ticks on each animal was counted at 14-day intervals. The results obtained from that study led to the following conclusions:

- *B.microplus* larvae are found on the pastures throughout the year

- the longest incubation period of eggs and longest survival of larvae are observed in the progeny from females which drop on the pasture between April and July, and March and June, respectively; whereas the shortest incubation period of eggs and survival of larvae are observed in the progeny from females dropping on pastures between September and March, and October and December, respectively

- the maximum duration of the non-parasitic stage of the tick life cycle is observed in progeny from engorged females dropping on pastures in April, May and June; whereas the minimum duration is observed in progeny from engorged females dropping on the pasture in October, November and December

- the maximum duration of the non-parasitic stage of the tick life cycle is 6.3 months and occurs in May; whereas the minimum duration is 3.8 months and occurs in November

-all the non-parasitic stages of the life cycle are affected by climatic conditions, particularly temperature; the duration of these stages is increased during the winter and decreased during the summer

- infestations with *B.microplus* are detected throughout the year, with the highest rates occurring between February and July

- the natural detachment of engorged females occurs more frequently in the afternoon and starts on day 20 after infestation, peaking on day 22

- under natural field conditions, four generations of ticks occur per year, and the number of ticks completing each generation is affected by climatic factors

- the time of the year most appropriate for the initiation of a control programme is during the first tick generation which occurs in November.

In a recent report by Honer and Gomes (1992), the potential number of generations of *B.microplus* ticks per year was estimated for different parts of Brazil. This was achieved by

adapting field observations to the Climex programme. The resultant mapping indicated that in the south of the country the conditions are appropriate for the development of up to three generations per year, except in the extreme south (Santa Vitoria do Palmar), where ticks can survive experimentally but are not normally found in the field. The central, southeast and west regions have appropriate conditions for the development of up to four tick generations per year.

The conventional and most widely used method of tick control is based on acaricide dipping according to the intensity of engorged female *B.microplus* infestations. In general at least 9 to 10 dippings are required during the year. Recently Magalhaes & Lima (1991) reported a very efficient strategic tick control regime for one farm in the State of Minas Gerais, in which the animals were only dipped during the rainy season (October - March) using pyrethroids on six occasions at 21 day intervals. This regime reduced tick infestation to an average of 0,29 engorged females per animal over a period of 1 year. However, such low rates of tick infestation mean that transmission rates of *Babesia* spp may fall below those required to maintain enzootic stability (Mahoney, 1977).

Tick and tick-borne disease control methods need to be considered very carefully in order to keep tick infestation rates high enough to maintain enzootic stability and minimise the risk of disease outbreaks, while minimising the direct damaging effects of ticks.

Despite the fact that, in some endemic situations, age resistance means that most calves become infected and develop immunity without disease, it appears that on Brazilian farms calves are the most likely group to suffer from clinical disease and death particularly from *B. bovis* and *A.marginale*.

The babesias were first diagnosed in Brazil by Fajardo (1901), in blood smears taken from imported cattle. This was followed by reports in 1908 by Dr Carini, and in 1913 by Drs Dupont, Miranda and Horta (all cited by Fonseca & Braga, 1923). In 1914 Drs Horta and Miranda presented a report about American babesias, during the 10th International Congress of Veterinary Medicine in London. In 1917, Dr Dupont described a visceral form of babesiosis responsable for high mortality rates in calves born in Brazil (cited by Fonseca & Braga, 1923).

In a book published in 1923, Fonseca and Braga covered the various aspects involved in the bovine babesiosis in Brazil, such as its transmission, clinical symptoms, pathogenesis, diagnosis and prophylaxis. At that time, they had already classified the disease into two forms according to the agent involved. The so-called *Piroplasma bigeminum*, which exhibited high polymorphism, was responsible for a 'peripheral' form of disease, characterised by easily detectable parasites in the peripheral blood, depression, constant fever, haemoglobinuria,

tachycardia, for which treatment with Trypan Blue was effective. *Piroplasma argentinum*, which was two or three times smaller than *P.bigeminum*, had a preference for parasitising red blood cells of internal organs, particularly the kidneys, and was responsible for a 'visceral' form of disease in which the parasites were rarely detected in blood smears. When haemoglobinuria was present it occurred at a late stage of the disease and indicated an unfavourable prognosis. Treatment with Trypan Blue was not effective against this visceral form of babesiosis (Fonseca & Braga, 1923). These authors also reported both successes and failures in a series of immunisations of imported cattle using inoculation of blood from indigenous donors. In order to minimise the loss of animals due to severe clinical disease, they proposed that the blood for inoculation should be 'attenuated' for 24 hours on ice.

The State of Minas Gerais has the largest cattle population in Brazil, with approximately 20 million animals. In a survey carried out in 30 farms in a district of Minas Gerais State, tick-borne diseases were cited as the second most frequent disease of calves, after diarrhoea (Leite, 1982). In blood smears from 113 calves selected randomly in those farms, *A.marginale* was found in 85% and *B.bigemina* in 36%.

Despite the economic importance of tick-borne diseases, few serological surveys have been carried out in Brazil. Those which have been done used the IFAT. Variable figures for seroprevalence have been reported. In a serological survey using 315 samples from adult cattle (> 2 years old), carried out in a region of Minas Gerais State (Zona da Mata) which produces about 1,600,000 litres of milk per day, variable prevalence rates were found for each micro-region sampled. The figures of seropositivity obtained for *B.bovis* varied from 59.5 to 88.9%, whereas for *B.bigemina* variation between 61.7 and 97.9% was observed (Patarroyo-Salcedo et al, 1987).

In another serological survey carried out with samples from Zebu cattle raised in the 'cerrado' region (State of Mato Grosso do Sul), in which a total of 667 serum samples from 3-10 year old cows were analysed by IFAT for detection of antibodies against *B.bovis* and *B.bigemina*, average prevalence rates of 19.0 and 12.9% were reported for *B.bovis* and *B.bigemina* respectively. Those figures are considerably lower than in the Minas Gerais survey, suggesting endemic instability (Madruga et al, 1983).

However the epidemiological value of the results from both studies is very limited since those surveys tested serum samples from adult animals and no information was collected regarding animal husbandry, breed, management, tick control, incidence of disease, mortality, etc. Also it is well recognised that cross-reactivity between *B.bovis* and *B.bigemina* is a severe limitation of the particular serological test used. The two infections appear to be distributed throughout the country, and there are suggestions that *B.bovis* infections are more severe than *B.bigemina* causing higher mortality in calves. However, more comprehensive

epidemiological studies are needed to determine the real situation of the two infections and their effect on cattle production. Based on these, appropriate and specific methods of control could be proposed for each situation.

Therefore reliable, specific and sensitive tests are needed which are able to discriminate between antibody responses to the different parasites involved, to determine the reality of the epidemiological situation. Despite the fact that little is known about the epidemiology of bovine babesiosis in Brazil, attempts to develop a Brazilian live attenuated vaccine were started in 1987 in a governmental research institute (EMBRAPA - CNPGC), by Kessler and co-workers, who isolated pure field strains of each parasite by tick infestation of splenectomised calves (Kessler et al, 1987a). The *B.bovis* strain was then rapidly passaged through 16 splenectomised calves, and the *B.bigemina* strain was slowly passaged through intact calves, with the aim of producing attenuation (Kessler et al, 1987b). A preliminary test of the pathogenicity and immunogenicity of these potentially attenuated strains showed that the *B.bovis* strain was of very low virulence, since no clinical disease was observed after vaccination. However the *B.bigemina* strain appeared to remain virulent, since three out of five calves showed clinical disease after immunisation (Kessler et al, 1987b). The test also showed that the two strains were highly immunogenic and protected the animals against challenge with the corresponding virulent homologous strain. However, these attenuated vaccines are not yet available on the Brazilian market.

The only commercial vaccine against bovine babesiosis available in Brazil is a chilled live vaccine produced in the State of Rio Grande do Sul by a private laboratory (Hemopar), which uses attenuated strains of both parasites from Uruguay (C.Arteche, personal communication). However, the vaccine production is only enough to satisfy the local demand, with farmers in the other states awaiting the implementation of production of an effective vaccine on a large scale basis.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 ORIGIN/SOURCE OF *BABESIA* PARASITES

Three stocks of *B.bovis* were used throughout the study: i) Mexico, a Mexican isolate (Smith et al, 1978; Erp et al, 1978) and ii) Kwanyanga, a South African isolate (Taylor & McHardy, 1979), both kindly donated to CTVM by Dr N. McHardy of Wellcome Research Laboratories, Beckenham, England, in 1982 and 1983 respectively, and iii) Lismore, an Australian isolate (Kahl et al, 1982b), kindly donated to CTVM by Dr S. Taylor, Stormont, Northern Ireland, in 1987. All three stocks were maintained at CTVM as blood or culture stabilates and had been cultured *in vitro* on several occasions (Conrad, 1983; L. Bell-Sakyi, personal communication).

Three stocks of *B.bigemina* were used throughout the study: i) Zaria, a Nigerian isolate, kindly donated to CTVM by Professor Uilenberg, University of Utrecht, Netherlands, in 1986; ii) Muguga, a Kenyan isolate, kindly donated to CTVM by Dr S. Williamson, National Veterinary Research Centre, Kenya Agricultural Research Institute, Muguga, Kenya, in 1990 and iii) Mexico, a Mexican isolate (Vega et al, 1985a) kindly provided as a growing culture by Professor E. Canning, Imperial College, London, in 1992. The Muguga and Zaria stocks were maintained at CTVM as blood stabilates while the Mexican stock had been cultured continuously *in vitro* and was maintained as culture stabilates.

3.2 *IN VIVO* INFECTIONS

A series of 12 calves, some of which were splenectomised, were infected with either *B.bovis* or *B.bigemina* and challenged one or more times, with a minimum period of 3 weeks between inoculations, with a *Babesia* stock using *in vitro* cultured material, blood stabilates or, on one occasion, infected ticks (see Table 3.1 and Appendix 7).

3.2.1 Splenectomy

Calves to be splenectomised were anaesthetised using 3 mg/kg of alphaxolane/alphadolone acetate (Saffan, Pitman-Moore), intubated and maintained with 1.5-2.0% halothane gas anaesthetic (Fluothane, Imperial Chemical Industries). An approximately 20 cm long skin incision was made on the left side, 2 cm caudal and parallel to the 13th rib. The external abdominal oblique, internal abdominal oblique, transverse abdominus and peritoneum were incised and the spleen exteriorized. The splenic artery and vein were ligated using No 1 chromic cat gut, the vessels were then severed and the spleen removed. The peritoneum and abdominal musculature were sutured using No 1 cat gut in a continuous suture. The skin was closed using No 2 nylon.

Table 3.1 Experimental infection of calves with *B.bovis* and *B.bigemina*

Calf No	Initial inoculum	Parasite (stock)	Challenge 1	Challenge 2	Challenge 3
396 [a] **	CS	*B.bovis* (Mexico)	*B.bovis* (Mexico) day28 (TC)	*B.bovis* (Mexico) day56 (TC)	*B.bovis* (Mexico) day90 (BS)
P78 [a] *	CS	*B.bovis* (Lismore)	*B.bovis* (Kwanyanga) day 21 (CS)	*B.bovis* (Lismore +Kwanyanga) day 42 (CS)	*B.bovis* (Lismore+ Kwanyanga) day 80 (BS + CS)
198 [b] *	BS	*B.bovis* (Kwanyanga)	———	———	———
P49 [b] *	FB	*B.bovis* (Lismore)	———	———	———
C11 [b] *	BS	*B.bovis* (Kwanyanga)	———	———	———
397 [a] **	BS	*B.bigemina* (Zaria)	*B.bigemina* (Zaria) day 62 (FB)	———	———
399 [a] **	BS	*B.bigemina* (Zaria)	*B.bigemina* (Zaria) day 29 (FB)	*B.bigemina* (Zaria) day 57 (BS)	*B.bigemina* (Zaria) day 98 (FB)
583 [b] **	BS	*B.bigemina* (Zaria)	*B.bigemina* (Zaria) day 35 (BS)	*B.bigemina* (Muguga) day76 (FB)	———
634 [b] **	IT	*B.bigemina* (Muguga)	———	———	———
U44 [b] *	BS	*B.bigemina* (Zaria)	———	———	———
7T [b] *	BS	*B.bigemina* (Zaria)	———	———	———
8T [b] *	BS	*B.bigemina* (Zaria)	*B.bigemina* (Zaria) day 21 (BS)	———	———

[a] intact
[b] splenectomised
* infected in previous experiments at CTVM
** infected for present study

BS blood stabilate
CS culture stabilate
FB fresh blood
IT infected ticks
TC tissue culture

3.2.2 Inoculum

When blood or culture stabilates were used as inoculum, 2 x 1 ml cryopreserved vials (see section 3.3.3) were thawed completely in a 37° C water bath and the contents of one vial was immediately inoculated intravenously and the other subcutaneously.

When cultured infected erythrocytes were used as inoculum, 12 to 15 ml of culture suspension, containing approximately 1.5 x 10^9 RBC/ml with an average parasitaemia of 5%, was centrifuged at 1,500 x g for 15 minutes at 4°C and the pelleted red blood cells (RBC) were washed by centrifugation with phosphate buffered saline (PBS) pH 7.2 (Appendix 1). The final pellet was resuspended in 3 volumes of PBS and half of the suspension was inoculated subcutaneously and the other half was inoculated intravenously.

Only one of the calves (calf 634) was infected by infestation with approximately 5,000 larval *Boophilus decoloratus* ticks, infected with *B.bigemina* (Muguga). The infected larvae were enclosed in cloth patches glued to both sides of the calf's neck, and allowed to feed until detachment of engorged females (21-23 days) (L Bell-Sakyi, personal communication). The remaining feeding ticks were destroyed by spraying the calf with amitraz (Taktic, Camco).

3.2.3 Monitoring of infection

Rectal temperature was taken daily and jugular blood was collected at least three times a week in a 5 ml vacutainer tube with disodium edetate (Becton Dickinson) for haematological analysis. The packed cell volume (PCV) was estimated by the microhaematocrit method (Schalm et al, 1975), and white blood cells (WBC) and RBC were counted using an electronic particle counter (model ZM, Coulter Electronics). For assessment of parasitaemia, thin films were prepared from both jugular blood and peripheral capillary blood taken from the tail tip. The smears were air dried, fixed with methanol for 2 minutes and stained in a 5%(v/v) dilution of Giemsa stock solution in Giemsa buffer, pH 7.2 (Appendix 1) for 40 minutes. The smears were then rinsed with buffer, air dried and examined under oil immersion at x 500 or x 1000 magnification using a light microscope (Leitz Wetzlar). At least one thousand RBC were counted in each smear and the parasitaemia (percentage infected RBC) was calculated. Results from infection monitoring are presented in Appendix 7.

Serum samples were collected on day 0 and at two week intervals for a maximum period of 16 weeks. Blood was collected into sterile plain vacutainer tubes (Becton Dickinson) and allowed to clot at 37° C overnight. The sera were centrifuged at 2,500 x g for 30 minutes at 4° C and stored at -20° C until required.

3.3 *IN VITRO* CULTURE TECHNIQUES

3.3.1 Initiation of *B.bovis* cultures

When the parasitaemia in blood smears from infected calves had reached 0.1%, cultures were initiated following minor modifications (L Bell-Sakyi, personal communication) of standard techniques (Levy & Ristic, 1980; Vega et al, 1985a). Approximately 40 ml of blood were collected aseptically by jugular puncture, defibrinated by shaking with glass beads and centrifuged at 1,000 x g for 10 minutes at 15° C. The plasma, buffy coat and the upper 20% RBC were discarded. The remaining RBC were used to prepare a 10% v/v suspension in complete medium M199 (see Table 3.2), supplemented with additional 2 mM L-glutamine. Culture vessels were inoculated to give a depth of 6-8 mm (i.e. 0.2 ml per well of a 96 well flat bottomed microtitre plate (Nunc); 1.25 ml in a 2 cm^2 well of a 24-well plate (Costar); 5 ml in a 25 cm^2 flask (Nunclon) incubated vertically; 12-15 ml in a 25 cm^2 flask incubated horizontally and 40 ml in a 75 cm^2 flask (Costar) incubated horizontally). Culture vessels were incubated at 37° C under an atmosphere of 5% CO_2 in air. In each experiment, cultures were incubated under the same conditions unless otherwise specified.

3.3.2 Maintenance of cultures

Culture medium was changed daily by carefully removing between one half and four-fifths of the overlying medium without disturbing the RBC layer, and replacing an equal volume of fresh medium.

Subcultures were carried out every 2-3 days by removing approximately four-fifths of the culture suspension and replacing the volume removed with fresh uninfected 10% RBC suspension.

Three adult cows were used as donors of uninfected RBC and serum: Limousine/Jersey crossbreeds No 16 (five-year-old) and No 110 (six-year-old), and Split Ear, a one year-old pure-bred Jersey, maintained at CTVM.

For uninfected RBC suspension preparation, blood from donor animals was collected by jugular puncture, defibrinated by shaking with glass beads and centrifuged at 1,000 x g for 10 minutes at 15° C. The plasma, buffy coat and the upper 20% RBC were discarded. The remaining RBC were used to prepare a 10% v/v suspension as previously described in 3.3.1. RBC suspension was stored at 4° C for up to 1 week after collection.

For serum production, blood was collected into plain sterile vacutainer tubes (Becton Dickinson) and allowed to clot at 37 ° C overnight. The sera were separated from clotted

blood by centrifugation at 2,500 x g for 30 minutes at 4° C and stored at 4° C for up to 1 week after collection.

Cultures of *B.bigemina* (Mexico) (Vega et al, 1985a), obtained as a growing culture suspension from Imperial College, were maintained under the same conditions used for *B.bovis* cultures, except that the culture medium was modified (see Table 3.2).

3.3.3 Assessment

Parasitaemia in cultures was estimated by preparing thin smears from representative samples taken from individual cultures after mixing the RBC suspension following medium changes. Parasite morphology was assessed from examination of Giemsa stained cytocentrifuge smears. Smears were made by centrifuging an aliquot of approximately 30 µl of culture suspension per well of a cytocentrifuge (Cytospin, Shandon Southern Instruments) onto microscope slides at 1,000 x RPM for 5 minutes. The smears were fixed, stained and examined as described in section 3.2.3.

3.3.4 Cryopreservation

Cultures, with an average parasitaemia of 5%, were centrifuged at 1500 x g for 10 min at 4° C and the resultant RBC pellet was resuspended in 1 ml of ice-cold medium M199-H (*B.bovis*) or M199-IC (*B.bigemina*) (as described in Table 3.2), for every 10 ml of original culture suspension. An equal volume of an ice-cold 10% (v/v) solution of dimethyl sulphoxide (DMSO, Analar, BDH) in culture medium was added dropwise with agitation to give a final concentration of 5% DMSO. The cell suspension was then divided between 1 ml cryotubes (Nunc) pre-cooled to -20°C and transferred immediately to the gas phase of a liquid nitrogen refrigerator.

3.3.5 Resuscitation

A vial was removed from the liquid nitrogen refrigerator and immediately immersed in a 37° C water bath until the contents had thawed completely. The contents were immediately mixed with 15 ml of warm (37° C) M199 and centrifuged at 2500 x g for 10 min at 15° C. The supernatant was discarded, the pellet resuspended in 1 to 5 ml of a 10% uninfected RBC suspension (see section 3.3.1) and the resulting suspension used to inoculate culture vessels. Cultures were incubated at 37° C under a reduced O_2 atmosphere of 4% O_2, 5% CO_2, 91% N_2 for the first few days after resuscitation. As soon as the parasites showed good multiplication rates they were changed to an atmosphere of 5% CO_2 in air.

Table 3.2 - Culture Media

Medium	Composition	Use
complete M199-H	M199 [1], 40% NBS	standard *B.bovis* culture
complete M199-IC	M199 [2], 40% NBS	standard *B.bigemina* culture
RPMI-1640-ML	RPMI-1640[3],without methionine, 5% NBS	metabolic labelling of *B.bovis* and *B.bigemina*
M199-HS	M199 [1], 5% NBS	harvesting of *B.bovis* culture supernatant
M199-ICS	M199 [2], 5% NBS	harvesting of *B.bigemina* culture supernatant

[1] with Hanks balanced salt solution and 25 mM HEPES (Gibco Cat. No 041-02350H)
[2] with Hanks balanced salt solution (Gibco Cat. No 041-01151H) to which 15 mM HEPES was added
[3] selectamine kit (Gibco)
NBS - normal bovine serum

3.4 SERA AND MONOCLONAL ANTIBODIES

3.4.1 Experimental sera

Bovine sera raised experimentally against each parasite stock were used for immunological studies. Serum samples were collected on day 0 and at specific times after primary infection and challenge of the calves described in section 3.2 and were stored at -20° C until required. Serum samples from calves P78, 198, P49, C11, U44, 7T and 8T had been produced during previous experiments carried out at CTVM and were stored at -20°C in a bank of serum samples in the Protozoology Section.

3.4.2 Field sera

Serum samples from Brazil, kindly provided by Dr R. H. Kessler (Empresa Brasileira de Pesquisa Agropecuaria - EMBRAPA - Centro Nacional de Pesquisa de Gado de Corte, Brazil), were collected at a farm located in Campo Grande, Mato Grosso do Sul.

Serum samples from Mozambique, kindly provided by Mr L. Neves, Universidade Eduardo Mondlane, Maputo, Mozambique, were collected from Xai-Xai, Chokwe and Chiango, in the southern part of Mozambique.

Serum samples from Malawi, kindly provided by Mr M. Edelsten, Livestock Disease Evaluation Project, Central Veterinary Laboratory, Lilongwe, Malawi, were collected from an area within 30 km of Lilongwe.

All field serum samples were collected from cattle that had presumably been exposed to tick-induced field infections with the respective stocks of *B.bovis*, *B.bigemina* and *Anaplasma marginale* native to each country. Cattle from Malawi and Mozambique may also have been exposed to field infections of *Theileria* sp and *Cowdria ruminantium*.

3.4.3 Monoclonal antibodies

A panel of 11 monoclonal antibodies (McAb) raised against the Kwanyanga stock of *B.bovis* was kindly provided as ascites by Dr S. Crowe, Wellcome Biotechnology, Beckenham, Kent. These were used in the immunochemical characterisation of stocks of *B.bovis* (described in Chapters 6 and 7).

3.5 INDIRECT FLUORESCENT ANTIBODY TEST (IFAT)

For antigen slide preparation, *Babesia* culture suspensions were centrifuged at 1,000 x g for 10 minutes at 4° C. The supernatants were discarded and erythrocytes washed twice with PBS by centrifugation. The volume of packed erythrocytes was estimated and approximately 2 volumes of a freshly prepared solution of 1% (w/v) bovine plasma albumin (BPA, fraction V, Sigma) in PBS was added. After gentle mixing the RBC suspensions were kept on ice and used to prepare antigen smears on glass microscope slides. Antigen smears were air dried, fixed in acetone for 15 minutes, air dried again and finally wrapped in tissue paper. The wrapped antigen slides were stored at -20° C in polythene bags containing silica gel for periods up to 1 year.

When required, the slides were allowed to thaw out in polythene bags with silica gel for 30 minutes at 4° C and a further 30 minutes at room temperature. Three rows of six test wells each were then drawn on each slide with a diamond pencil making 18 wells per slide. Antigen slides were washed three times in PBS, drained and allow to dry before use.

Starting from a 1 in 40 dilution, five four-fold dilutions of each polyclonal serum in PBS were applied to test wells and the slides were incubated for 30 minutes at room temperature in humid chambers. In the case of each McAb only two dilutions, 1 in 10 and 1 in 100, were tested. Each slide included three control wells, one each of positive serum, negative serum and PBS. After the slides had been washed three times in PBS, the appropriate anti-species fluorescein isothiocyanate labelled conjugate (FITC) (Sigma) was applied to each well, at the manufacturer's recommended dilution in PBS, and the slides incubated for a further period of 30 minutes. The slides were then washed in PBS as before, air-dried, mounted (see Appendix 1), covered with a 22 x 64 mm coverslip and examined using a x 54 oil immersion objective on a microscope equipped for incident excitation of FITC (Leitz-Orthoplan, Wetzlar).

3.6 SODIUM DODECYLSULPHATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

3.6.1 Preparation of samples

3.6.1.1 *B.bovis*

Each stock of *B.bovis* and a negative control of uninfected RBC suspension were cultured *in vitro* (section 3.3). Approximately 400 ml of each culture (10% v/v RBC suspension) were centrifuged at 1,600 x g at 4°C for 15 minutes. The pelleted cells were washed twice by centrifugation with PBS as described above and the packed RBC were resuspended in 2 volumes of PBS and passed through 50 cm x 4 cm glass columns containing CF-11 cellulose

powder (Whatman) at a proportion of 1.25 g per ml of packed RBC to remove WBC (Melrose & Brown, 1979). The columns were eluted with 200 ml PBS and fractions of approximately 100 ml were collected. Ten µl of each fraction were applied to a plain microscope slide, mixed with 10 µl of a 0.01% (w/v) acridine orange solution in McIlvane's buffer (Appendix 1) and covered with a 22 x 22 mm coverslip. Slides were examined on a microscope equipped for incident excitation of acridine orange to check for the absence of WBC. The acridine orange stains the DNA of WBC green. WBC-depleted RBC suspensions were then centrifuged as described above, the supernatants discarded and the volume of packed RBC measured. Hypotonic lysis of uninfected RBC was achieved according to a modification of the method of Kahl et al (1982a). Twenty five volumes of 0.5%(w/v) KCl (at 4°C) were added to the packed RBC and immediately mixed thoroughly. Suspensions were agitated briefly every 30 seconds until completion of lysis (generally 5 minutes), indicated by the changing of colour from bright to dark red. A calculated volume (the volume of packed RBC multiplied by the constant 1.57) of 10.9% (w/v) KCl was added to return the solution to normal bovine RBC isotonicity. Suspensions were then centrifuged as previously described and pelleted infected RBC (iRBC) were washed twice by centrifugation with PBS. The supernatants were discarded and the remaining packed iRBC were lysed by exposure to 5 volumes of 0.9%(w/v) NH_4Cl for 30 minutes at 4°C. The resulting free parasites and RBC membranes were washed with 50 ml of PBS and centrifuged as previously described. The final pellet was frozen as 200 µl aliquots until required.

3.6.1.2 *B.bigemina*

B.bigemina (Mexico) samples for electrophoresis were prepared by using concentrated iRBC obtained by continuous Percoll density gradient centrifugation as described in Chapter 5. The concentrated iRBC were washed three times with PBS by centrifugation (1,600 x g for 10 minutes at 4° C) in order to remove the Percoll solution. The final pellet was frozen as 200 µl aliquots at -80° C until required.

3.6.2 SDS-PAGE

Separation of proteins according to their molecular weight was performed by vertical SDS-PAGE under reducing conditions using the Laemmli buffer system (Appendix 1) (Laemmli, 1970) on either 7-20% gradient gels or homogeneous 10% gels.

Gels were prepared in vertical glass plates (160 mm x 180 mm) separated with 1.5 mm plastic spacers. Each gradient gel was cast using a gradient mixer containing 20 ml of each acrylamide solution (Appendix 1) and homogeneous gels were prepared with 40 ml of a 10%

gel solution. Resolving gel solutions of 7%, 10% and 20% and stacking gel solutions of 4.5% acrylamide (Appendix 1) were prepared and stored as 20 ml and 10 ml aliquots respectively at -20°C. As required, one aliquot of each appropriate resolving gel solution and one aliquot of stacking gel solution were thawed in a water bath at 37°C. To each 20 ml of resolving gel solution, 26 µl of freshly prepared 10% (w/v) ammonium persulphate solution and 6 µl of TEMED (BDH) were added and the gels were allowed to polymerise at room temperature for approximately 2 hours. After polymerisation of the resolving gel, 100 µl of ammonium persulphate and 6 µl of TEMED were added to 10 ml stacking gel solution which was immediately overlaid. A teflon comb was inserted to produce 15 sample wells (60 µl/well) and the gel allowed to polymerise for approximately 30 minutes.

Samples of *B.bovis* and *B.bigemina* (as described in 3.6.1) were thawed out and approximately 50 µl of each sample were loaded per lane.

Electrophoresis was conducted overnight at room temperature, at 100 V constant voltage which corresponded to a starting current of 18-19 mA per gel.

In some experiments the Protean II mini-gel system (Bio-Rad) was used either with pre-cast (Bio-Rad) or home-made 10% homogeneous gels. All gels contained 10 sample wells. Twenty five µl of sample were loaded per well and the gels were electrophoresed at a constant 200 V for a period of 45 minutes.

To enable calculation of protein molecular weights, a sample well containing a set of low molecular weight markers (Pharmacia or Sigma) was included in each gel.

3.6.3 Protein detection

Following electrophoresis, gels were stained with either Coomassie Brilliant Blue R-250 or silver nitrate. All solutions were prepared with highly purified water (18 megahoms, as described in Appendix 1).

Coomassie blue staining was performed by soaking the gels in 25% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie blue R-250 for 1 hour. Gels were then destained in 25% (v/v) methanol, 10% (v/v) acetic acid until the background was clear (generally 2 hours at room temperature) and then photographed through a Wratten 11 filter (Appendix 3).

Silver staining was conducted by fixing the gels in a solution of 25% (v/v) methanol, 10% (v/v) acetic acid and then in a 5% (v/v) methanol, 7% (v/v) acetic acid solution for 30 minutes each. Gels were soaked in 10% (v/v) gluteraldehyde for 30 minutes and then either washed with distilled water several times over a period of 2 hours or left overnight in distilled water to remove all traces of gluteraldehyde. Gels were stained in 0.1% (w/v) silver nitrate

for 30 minutes and, after removal of the staining solution, rinsed with developer (50 µl of 37% (w/v) formaldehyde in 100 ml 3% (w/v) Na_2CO_3). Fresh developer solution was added and development continued until staining was optimal. The reaction was stopped with 10 ml of a 2.3M citric acid solution and gels were photographed through a Wratten 61 filter (Appendix 3).

3.7 WESTERN IMMUNO-BLOTTING

Following the method of Towbin and co-workers (Towbin et al, 1979), proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes (blots) using a semi-dry electroblotter (Sartorius) at a constant current of 0.8 mA/cm^2 of gel. The strip of membrane corresponding to the molecular weight markers on the gel was cut from the blot and stained with 0.1% (w/v) amido black for approximately 15 minutes. The strip was then destained until the bands were clearly visible (generally for 30 minutes), rinsed with distilled water, dried and stored protected from light at room temperature until required for molecular weight estimations.

Free binding sites on the nitrocellulose blots were blocked for 4 hours with blocking buffer (Appendix 1). Blots were then incubated overnight with either bovine polyclonal serum or mouse monoclonal antibody ascites fluid, both diluted 1:100 in blocking buffer. After removing the antibody solutions, the blots were washed 7 x with PBS over a 2 hour period. Blots were then incubated for 2 hours with appropriate dilutions of horseradish peroxidase-conjugated anti-bovine or anti-mouse immunoglobulin (Sigma) in blocking buffer. After removing the conjugate solutions, the blots were washed 7 x with PBS. Antigen-antibody complexes were visualised by incubating the blots in a peroxidase substrate solution containing 60 mg 4-chloro-1-naphthol in 20 ml of ice-cold methanol which was mixed immediately before use with 100 ml Tris buffered saline pH 7.0 (TBS) (Appendix 1) to which 60 µl ice-cold hydrogen peroxide had been added. As bands became visible, the reaction was stopped by removing the substrate solution and washing the blots 2 x in distilled water. The membranes were allowed to dry before they were photographed (Appendix 3).

3.8 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

ELISAs were carried out according to Woodford and co-workers (Woodford et al, 1990) with minor modifications. Briefly, 100 µl of antigen diluted in coating buffer (Appendix 1) were dispensed into each well of a microELISA plate (Dynatech Ltd, M129 A), and the plates incubated overnight at 4^{o} C. The plates were washed for 3 x 1 minute washing cycles with PBS containing 0.05% Tween 20 (PBST) using a commercial plate washer (Dynatech Ltd).

Each serum sample was diluted in PBST and 100 µl of each sample was dispensed in duplicate into the appropriate wells. Each plate had a set of 16 control wells: four wells of *B.bovis* serum (taken from calf 396 on day 56 after infection), 4 wells of *B.bigemina* serum (taken from calf 399 on day 57 after infection), 4 wells of a pre-infection serum (taken from calf 634 on day 0) and 4 wells of PBST. The plates were incubated at 37° C for 30 minutes. Unbound antibodies were removed by washing the plates as described above. One hundred µl of anti-bovine peroxidase labelled immunoglobulin conjugate (Sigma) diluted in PBST as recommended by the manufacturers were added to each well and the plates incubated for 30 minutes and washed as described above. Peroxidase substrate buffer was prepared by dissolving one tablet of phosphate-citrate buffer with sodium perborate (Sigma) in 100 ml of distilled water. One tablet of 3,3'5,5'-tetramethylbenzidine (Sigma) was dissolved in 10 ml of substrate buffer and 100 µl were added to each well of the plate. The plate was incubated at 37° C for 10 minutes and the reactions were stopped by adding 50 µl of 2M H_2SO_4 to each well. The optical density (OD) of the contents of each well was read at 450 nm using a microELISA plate reader (Titertek Multiskan, Flow Laboratories).

CHAPTER FOUR

IN VITRO CULTURE STUDIES

4.1 INTRODUCTION

The development of continuous *in vitro* culture systems for *Babesia* parasites allowed the beginning of a new era of research into many aspects of bovine babesiosis such as morphology, physiology, immunology and chemotherapy which had previously been totally dependent on *in vivo* infections in experimental animals. Of the *Babesia* affecting cattle, only *B.divergens* infects a laboratory animal, the Mongolian gerbil (*Meriones unguiculatus*). It has not been possible to infect laboratory animals with *B.bovis* and *B.bigemina*; as a consequence, *in vivo* studies on these two species are limited to infection of susceptible cattle, usually splenectomised calves.

The most significant application of the *in vitro* culture system has been the production of immunogens without influence from the host immune system. Cultured parasites have been used as source of immunogens in various parts of the world including Venezuela (Montenegro-James et al, 1985), Mexico (Smith et al, 1981), Israel (Pipano, 1989) and Australia (Timms et al, 1983). The *in vitro* culture system developed for *B.canis* (Molinar et al, 1982) has been used to provide immunogens for immunisation against canine babesiosis in France, in a commercially available vaccine ("Pirodog", Merieux, France).

In vitro cultivation has also facilitated comparative studies on characterisation of isolates from different parts of the world and simplified cloning experiments, which in the past had been carried out using large numbers of animals (Gill et al, 1987a).

Due to the economic constraints of experiments involving *in vivo* infections, particularly the high costs of purchase and maintenance of animals and the associated labour, most of the *Babesia* material used throughout this study had to be provided by *in vitro* culture. In addition to economic limitations, the nature of certain experiments, in particular metabolic labelling for immunochemical analysis, required the provision of parasites continuously over a long period of time, making culture systems the most appropriate option as a source of parasites.

In the past, several attempts have been made at CTVM to culture *in vitro* an African isolate of *B.bigemina* (Zaria) without success (L.Bell-Sakyi, personal communication). This failure to establish a continuously growing culture system was the major limitation of the immunochemical investigation of *B.bigemina* isolates. Therefore, in the present study, further attempts were made to establish a *B.bigemina* culture system using the existing Zaria stock, a new isolate from Kenya (Muguga stock) and a third isolate, from Mexico, obtained from the Imperial College, London, (described in section 3.1) that had been adapted to culture (Vega et al, 1985a).

Three isolates of *B.bovis* from different continents, Australia, Africa and South America, (as described in section 3.1) had been grown in *in vitro* using the MASP system with minor modifications and held as frozen culture stabilates at CTVM (Conrad, 1983; L.Bell-Sakyi, personal communication), . The parasitaemias of cultures achieved at CTVM have been comparable to the ones reported in the literature (5 to 11%). However, these figures are still low, making the culture system expensive and often inappropriate if a large amount of parasite material is needed.

Thus, the experiments described in this chapter were designed to investigate and optimise *in vitro* culture conditions to exploit the culture systems as a reliable source of parasite material for use in later experiments on immunochemical characterisation of the stocks available. These included attempts to establish an *in vitro* culture system for *B.bigemina* and optimisation of the existing *B.bovis* culture system. The latter involved the establishment of cultures from low parasitaemia blood, cloning of one isolate, and incorporation of cell feeder layers into the cultivation system. This chapter also describes a series of experiments by which the effects on parasite growth of immune sera raised against *B.bovis* and *B.bigemina* were investigated.

4.2 EFFECT OF DIFFERENT SERA ON *B.BOVIS* CULTURE

The MASP system developed for cultivation of *B.bovis* (Levy & Ristic, 1980) has been described in section 3.3.

The source of the serum component of culture medium appears to be one of the most limiting factors in cultivation of *Babesia* parasites, since not all cattle provide serum that supports *in vitro* growth; it has been reported in the literature that adult bovine serum is preferable to calf serum for use in cultivation of *B.bovis* and that newborn and most bobby calf sera do not support *B.bovis* growth *in vitro* (Kellermann et al, 1988). Therefore, the first step in the establishment of a culture system has to be the identification of suitable serum donors.

Studies on passive transfer of immune serum to susceptible animals (Mahoney et al, 1979) and passive transfer of maternal immunity (Hall, 1960; Hall et al, 1968) give indirect evidence of a protective role of antibodies in *Babesia* infections. On the other hand, attempts to correlate total antibody levels with immune status have failed (Mahoney, 1964; Callow et al, 1974), suggesting that not all antibodies are protective.

In endemic areas where *Babesia* infections are widespread with most of the adult animals having circulating specific antibodies, the identification of a serum donor which does not carry anti-babesial antibodies may constitute the major limitation for the maintenance of *in vitro* parasite growth.

With this in mind, the experiments described in this section were designed to evaluate the effect of immune sera on *in vitro* growth rates of *B.bovis*.

4.2.1 Experimental design

Growing culture suspensions of *B.bovis* (Mexico and Lismore stocks), showing a parasitaemia of 5% in thin smears, were used as source of infected erythrocytes to initiate cultures. The culture suspensions were subcultured into a 96-well plate at a 1 in 5 dilution with fresh 10% RBC suspensions prepared in M199-H, supplemented at 40% with one of each of the following sera: (for details of calf infections see Table 3.1)

animal No/ infection	age at infection	serum collection (days post-infection)	homologous titre (IFAT)
396 *B.bovis* (Mexico)	5 months	21 69	1 in 640 1 in 2560
397 *B.bigemina* (Zaria)	5.5 months	21 69	1 in 640 1 in 640
399 *B.bigemina* (Zaria)	5.5 months	36	1 in 2560
590 uninfected	4.5 months	----	negative
110 uninfected	6 years old	----	negative

All serum samples were used fresh (none of them had been frozen prior to addition to the culture medium). For serum separation, blood samples were aseptically collected by jugular puncture, on the day before the initiation of cultures, allowed to clot at 37°C overnight, centrifuged at 2,500 x g for 30 minutes at 4°C and stored at 4°C for up to one week.

Three replicate wells (200 µl/well) were set up for each culture medium tested and the plates were incubated under 5% CO_2 in air. The overlying medium (~ 150 µl) in each well was replaced with the same medium daily, and smears were also made daily up to day 6. Parasitaemias were estimated by counting 1,000 RBC in each smear and each well was subcultured (1 in 3 dilution) with the corresponding fresh 10% RBC suspension on day three.

The medians of counts of each set of replicates were compared statistically using the Chi-square test for homogeneous frequencies (Fowler & Cohen, 1992); this comparison was made for the two cycles of parasite growth separately, i.e. for the first phase of exponential growth (first three days) and for the second phase (three days after subculture).

4.2.2 Results

The counts obtained from each well of *B.bovis* (Mexico) culture, over the period of 6 days are presented in Appendix 2 and the medians of each replicate set are presented in Fig 4.1.

There was no significant difference ($p > 0.05$) between the medians of parasite counts from wells with culture media supplemented with *B.bovis* immune serum (from calf 396, taken at 21 days after infection) and NBS (control), suggesting that the specific antibodies against *B.bovis* did not inhibit parasite growth. The lack of inhibition was observed in both cycles of exponential growth. However, the medians of parasite counts from wells containing culture medium supplemented with serum from calf 397 (taken at 21 days after *B.bigemina* infection) were significantly lower ($p < 0.001$) than those obtained from control wells (supplemented with NBS) and from the cultures supplemented with serum from calf 396. These significant differences were observed in both cycles of parasite growth, suggesting that serum from calf 397, taken on day 21 after infection, did not support *B.bovis* growth *in vitro*.

The counts obtained from each well of *B.bovis* (Lismore) are presented in Appendix 2 and the medians of each set of replicates with each culture media are presented in Fig 4.2.

With this parasite there were no significant differences between medians of iRBC counts from any of the sera tested and the control counts in either cycle of parasite growth, suggesting that all the calf sera tested were as good as the NBS in supporting *in vitro* growth of *B.bovis*. In this second experiment no inhibitory effect was observed on *B.bovis* growth of immune serum (taken 69 days after infection) (Fig 4.2).

Fig 4.1 Medians of *B.bovis* (Mexico) iRBC in 1,000 cells in cultures using different sera.

Fig 4.2 Medians of *B.bovis* (Lismore) iRBC in 1,000 cells in cultures using different sera.

Fig 4.1

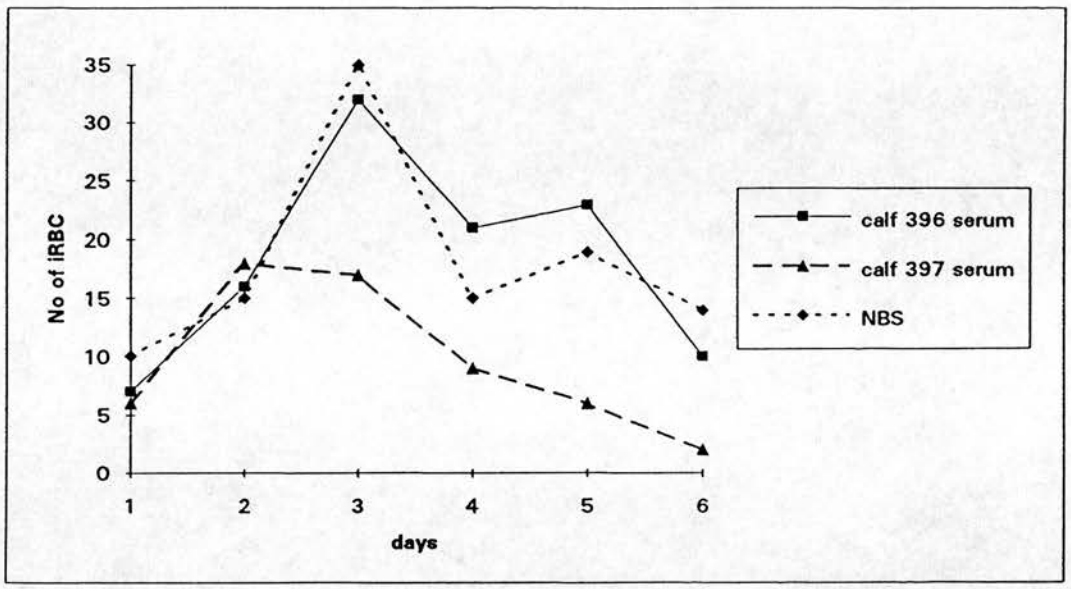
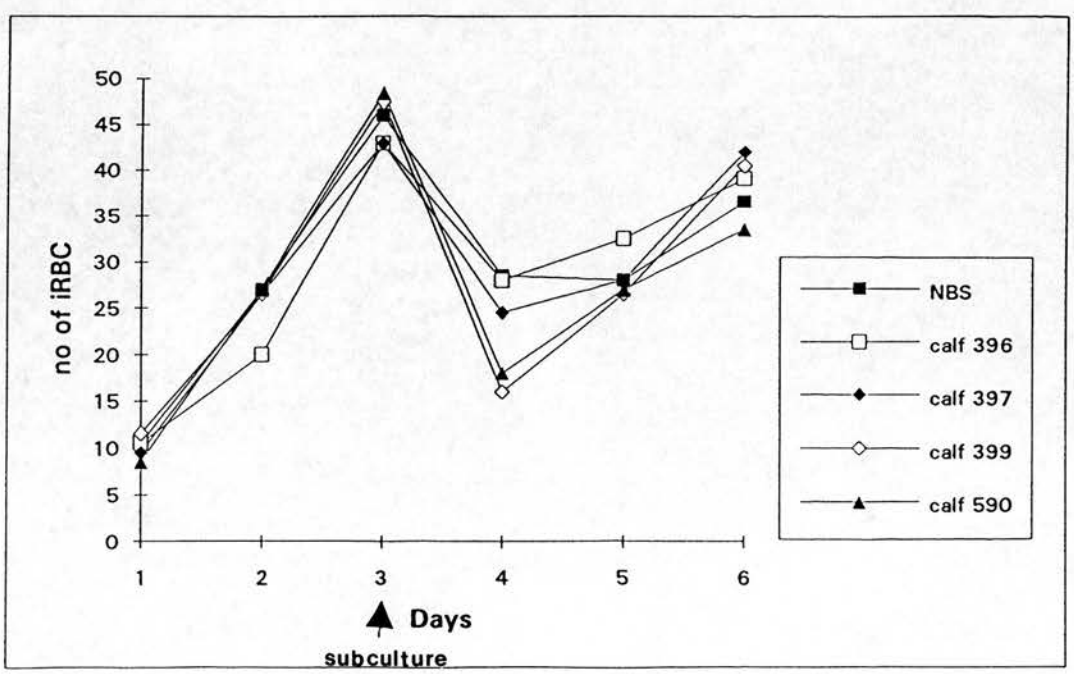

35
30
25
20
15
10
5
0
No of iRBC
1
2
3
4
5
6
days
calf 396 serum
calf 397 serum
NBS

Fig 4.2


50
45
40
35
30
25
20
15
10
5
0
no of iRBC
1
2
3
4
5
6
Days
subculture
NBS
calf 396
calf 397
calf 399
calf 590

4.2.3 Discussion

In the present study, sera from one uninfected calf and from 3 calves infected with either *B.bovis* or *B.bigemina* were substituted for NBS in culture medium and, with only one exception (serum from one calf taken after 21 days of infection with *B.bigemina*, used in the first experiment), all calf sera were found to be suitable for use in *B.bovis* cultures (Fig 4.1 and 4.2). These results contradict the reports of parasite inhibition *in vitro* by a factor in sera from young calves (Levy et al, 1982). However the higher inhibitory effects of young calf sera on *B.bovis* cultures reported by Levy and co-workers were obtained after 3 or 4 subcultures whereas, in the present study, the growth rates of parasites were evaluated over 6 days, including only one subculture during this period. In addition, all serum samples tested in the present study were taken from 4 to 6 month old calves and therefore it is possible that they had already lost the inhibitory factor.

On the other hand, the observed inhibitory effect of serum from calf 397 (taken 21 days after *B.bigemina* infection) on *B.bovis* growth *in vitro* (Fig 4.1) suggested an age effect, since serum from the same calf taken 48 days later (on day 69 after infection) did not have any inhibitory effect on parasite growth (Fig 4.2).

The inhibitory effect of calf sera on *B.bovis* growth has been associated with the presence of a factor independent of antibody, and responsible for calf resistance to severe babesiosis (Levy et al, 1982). In that report, it was demonstrated that the inhibitory factor present in calf serum is dialyzable, has a low molecular weight (< 14,000) and is lost as the calves age, since foetal calf serum gave the highest inhibition followed by new born calf serum (< 10 days old) and bobby calf serum (10 days to 3 months old).

All post-infection serum samples used in the experiments described here had been shown by IFAT to contain specific antibodies with titres of at least 1 in 640. However, the results obtained here led to the conclusion that those antibodies were not able to inhibit parasite growth in culture.

Previous studies have demonstrated the role of humoral immunity in protection against *B.bovis* infections; these included the passive transfer of immune serum conferring partial protection to susceptible calves (Mahoney et al, 1979). The subclass of antibody responsible for protection was determined to be IgG1 (Goff et al, 1982). Recently, Orinda and co-workers reported the failure of vaccinated cattle to produce interferon gamma, reinforcing the argument that immunity to *B.bovis* infection is correlated most strongly with a humoral rather than a cell-mediated immune response (Orinda et al, 1992a). In addition, it has been demonstrated that antibodies produced against protective antigens promote *in vitro* phagocytosis of *B.bovis* and *B.bigemina* (Parrodi et al, 1990; Jacobson et al, 1993) and

B.rodhaini (Parrodi et al, 1991); however complement did not enhance phagocytosis of infected erythrocytes or parasites alone. Based on these findings, it was expected that the immune sera tested in the present experiments, which had been shown to contain specific anti-*B.bovis* antibodies, would inhibit parasite growth *in vitro*. This was not observed.

The most unexpected result was the lack of parasite inhibition by serum from calf 396 taken on day 69 after primary infection, and after two challenges with the homologous stock of *B.bovis*, which had shown to contain high titres of specific antibodies (1 in 2,560) by IFAT. Although the serum was produced in a calf infected experimentally by inoculations of infected blood and may differ from sera derived from naturally tick-induced infections, the results obtained here indicate that it could be possible to use sera from sero-positive animals for *in vitro* cultivation of *B.bovis* in endemic areas.

The effect of post-infection calf sera on *B.bovis* growth *in vitro* has been previously examined at CTVM (L.Bell-Sakyi, personal communication), when a series of 10 frozen serum samples taken from calves on days 0 and 28 after infection (2 with *B.bovis*, 1 with *B.divergens*, 1 with *B.bigemina* and 1 with *T.annulata*) were tested and all of them had performances comparable to that of the frozen NBS control. There was, however, a significant increase in parasitaemia when the corresponding fresh NBS control was compared with all frozen sera, suggesting that none of the frozen sera (pre or post-infection calf sera and NBS) were as good as fresh NBS. Based on these findings, all serum samples tested in the present study were fresh (not previously frozen), which made impossible the comparison between the performance of pre and post-infection samples from the same animal.

The lack of parasite inhibition by calf immune sera in the present study might be related to the fact that the two stocks of *B.bovis* (Lismore and Mexico) used in the present experiments have been cultured *in vitro* for a considerable period of time, which may have resulted in selection of populations of parasites very well adapted to growth *in vitro* and able to survive more easily when subjected to adverse conditions.

Studies on *in vitro* inhibition of *Babesia* may be useful for understanding mechanisms related to parasite invasion and may help to identify immunoglobulins involved in protection. In that case however, specific immunoglobulins should be concentrated and purified before being incorporated into the cultures. Such studies have been carried out with cultured *P.falciparum* parasites, in which growth was inhibited by purified specific immunoglobulins from sera of individuals living in endemic areas (Brown et al, 1983).

4.3 USE OF FEEDER CELLS IN *B. BOVIS* CULTIVATION

Although the *in vitro* culture system for *B.bovis* has been successfully implemented at CTVM, the maximum parasitaemias achieved are still relatively low, usually ranging between 5 and 8%. Moreover, on some occasions, some of the cryopreserved stabilates have proved to be difficult to re-establish as a continuous culture (particularly Mexico and Kwanyanga isolates).

P.falciparum has been shown to exhibit better growth rates when cultured in association with other cells (Mazier et al, 1984; Phillips et al, 1987) and increased multiplication rates of asexual erythrocytic stages of this parasites have been reported when either new or established isolates were cultured in the presence of mouse peritoneal cells as a feeder cell layer (Trenholme & Phillips, 1989).

Previous work carried out at CTVM, showed that establishment of *B.bovis* cultures from infected calf blood were facilitated when bovine aortic endothelial cells (BAE) were incorporated into culture vessels as a feeder layer (Conrad, 1983).

Therefore, the experiments described in this section were designed to evaluate the incorporation of cell feeder layers in *B.bovis* cultures, with the aim of increasing parasitaemias of established cultures and improving the technique for resuscitation of stabilates. The study included a comparison between incorporation of mouse peritoneal wash cells (MPWC) and BAE cells in established cultures and also an evaluation of the effect of MPWC at different concentrations in cultures of the three stocks of *B.bovis* (Lismore, Mexico and Kwanyanga), using either established cultures or cryopreserved culture stabilates of each stock.

4.3.1 Preliminary study

A preliminary experiment was designed in which the effects of the two types of feeder cell layers on *B.bovis* cultures were compared.

MPWC were aseptically collected from adult female mice by peritoneal lavage with 10 ml ice-cold M199-H. The cells were washed twice in M199-H by centrifugation (400 x g for 5 min at 4^{o} C) and resuspended in complete culture medium (M199-H, 40% NBS). For this evaluation, three concentrations of MPWC were tested: 10^6, 10^5 or 10^4 cells/ml. The concentration of washed cells was estimated by haemocytometer count and the suspension was diluted with complete medium in order to give the required concentrations of MPWC.

BAE cells were taken from a primary culture maintained at 37°C in 5 ml of MEM (Eagle's), 10% foetal calf serum (FCS) in a 25 cm^2 flask under 5% CO_2 in air (Gray et al, 1985). Cells were harvested (according to A. Barns, personal communication) by adding 2 ml of a 0.1% trypsin-versene solution to the flask and incubating it at room temperature until the cell sheet was seen to be detaching from the flask. The cells were then transferred to a sterile centrifuge tube and washed twice with culture medium by centrifugation at 800 x g for 10 minutes. The resulting pelleted BAE cells were then resuspended in 5 ml of M199-H and used to inoculate wells.

B.bovis (Lismore) culture suspension (as a 20% RBC suspension in M199-H, 40% NBS, with parasitaemia of 1%) was used to initiate cultures in a 96-well plate.

Twelve wells (test wells) of a 96-well plate, three replicates for each condition, were set up as described:

Day -1(100 µl/well)	**addition on day 0 (100 µl/well)**
10^6 MPWC/ml	*B.bovis* culture***
10^5 MPWC/ml	*B.bovis* culture***
10^4 MPWC/ml	*B.bovis* culture***
BAE culture*	*B.bovis* culture***
CM**	*B.bovis* culture*** (control wells)

* diluted 1:3 with complete medium (CM)

** M199-H, 40% NBS

*** a 20% RBC suspension with 1% parasitaemia

Thus, each well contained a volume of 200 µl and a PCV of 10%. In the same plate, one well of each of the dilutions of MPWC (10^6, 10^5, 10^4/ml) and one well of BAE (200 µl/well) were set up as control wells without the addition of *B.bovis* culture.

The plate was incubated at 37° C in 5% CO_2 in air. Overlying medium was changed daily (100 µl/well) from day 0 in all wells. Parasitaemias in *B.bovis* culture wells were estimated daily for 4 days after medium changes by counting 1,000 RBC in thin smears stained with Giemsa as described in section 3.3.3. For evaluation of survival/growth of MPWC and BAE cells, each of the control wells was examined daily using an inverted microscope (Leitz Diavert).

Subcultures of the *B.bovis* wells were carried out on day 2 by gently pipetting and then removing 150 µl of culture suspension and replacing with fresh RBC suspension.

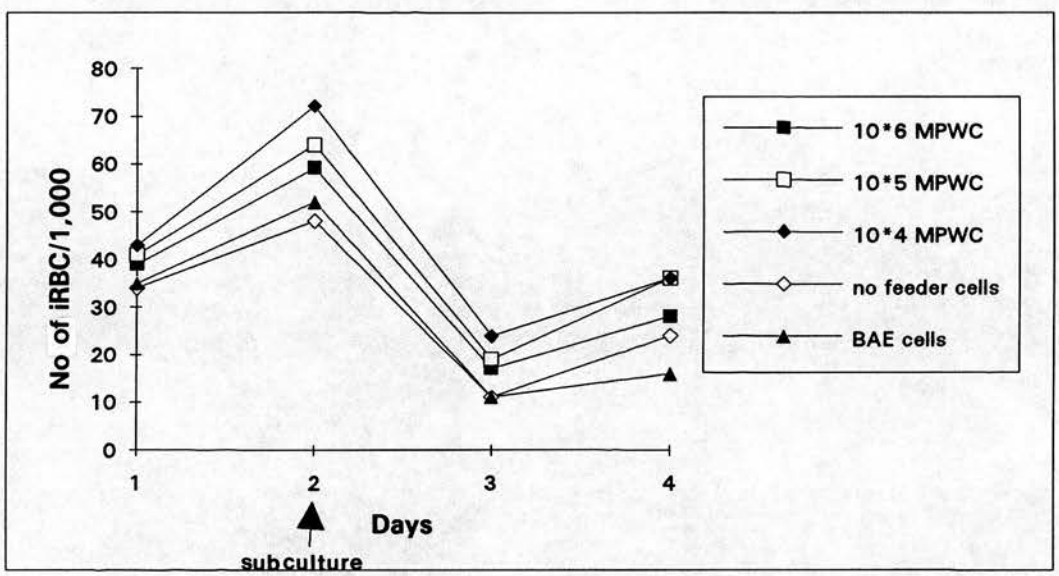
The medians of the counts obtained from each set of replicates of *B.bovis* cultures were statistically compared by the Chi-square test for homogeneous frequencies as previously described.

4.3.1.1 Results

There was no significant difference between the medians of counts obtained from the BAE cell feeder wells and the *B.bovis* control wells. However there was a significant increase in number of iRBC ($p < 0.001$) in cultures with all three concentrations of MPWC when compared with counts from the *B.bovis* control wells. When the different concentrations of MPWC were compared, no significant difference was found between them, suggesting that the three concentrations tested had a similar effect. The counts obtained from each well are presented in Appendix 2 and the medians of each set of replicates are presented in Fig 4.3.

Fig 4.3 Medians of *B.bovis* iRBC in 1,000 cells in cultures with MPWC or BAE as feeder cells.

Fig 4.3


80
70
60
50
40
30
20
10
0
No of iRBC/1,000
1
2
3
4
Days
subculture
10*6 MPWC
10*5 MPWC
10*4 MPWC
no feeder cells
BAE cells

4.3.2 Effect of mouse peritoneal wash cells (MPWC) on established cultures

For this study, two concentrations of MPWC were tested: 10^4 and 10^3 cells/ml, on established cultures of all three stocks of *B.bovis* (Lismore, Mexico and Kwanyanga).

The MPWC were harvested and prepared as described before, and diluted with complete medium in order to give the required concentrations.

Twelve wells of three 24-well plates (one for each stock) were set up on day -1 as described:

4 wells received 1 ml of a 10^4 MPWC suspension

4 wells received 1 ml of a 10^3 MPWC suspension

4 wells received 1 ml of medium (control)

The plates were incubated at 37°C under 5% CO_2 in air.

On day 0, the overlying medium of each well was carefully removed using a Pasteur pipette and 1.25 ml aliquots of *B.bovis* cultures (as 10% RBC suspensions with parasitaemia of 0.5%) were used to inoculate each well. The plates were incubated, medium changed (1ml/well) and parasitaemias estimated daily in smears from all wells by counting 1,000 RBC as described before.

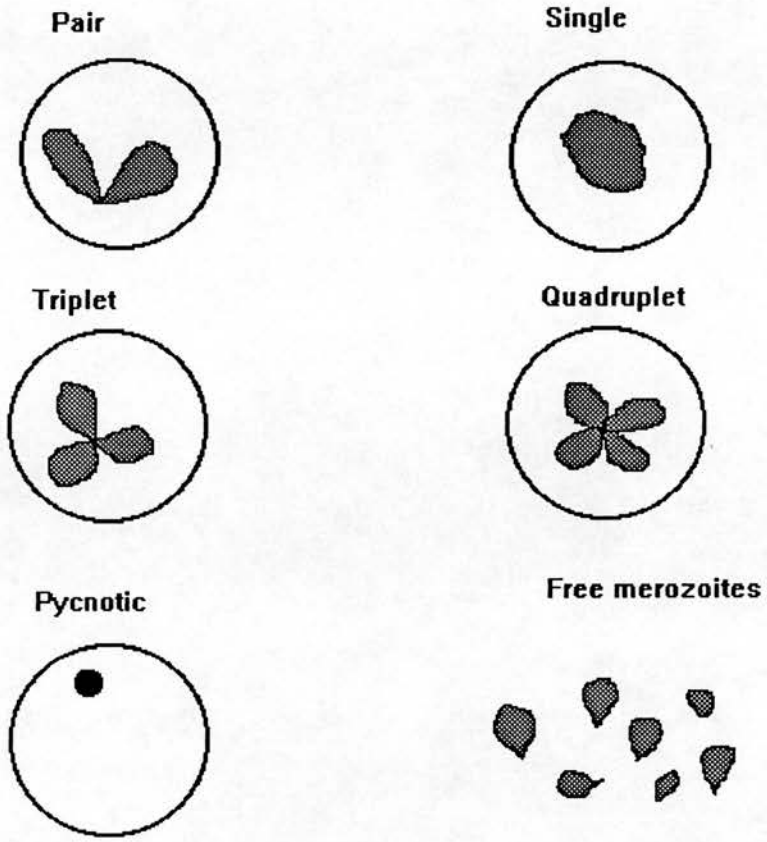
In addition to the parasite counts, a morphological analysis was also carried out. This was done by counting the different forms (single, pair, quadruplet, pycnotic and free merozoites, as described in Fig 4.4) present among 100 parasites in one smear from each treatment each day.

Subcultures (1 in 10 dilution) were carried out on day 3 into 12 new wells (in the same plate), which had previously been coated overnight with freshly collected MPWC or medium as described for day -1. Medium was changed and smears were made daily from each well until day 3 after subculture.

The medians of parasite counts from each set of replicates of each cycle of growth (before and after subculture) and the counts of the different parasite forms were compared by the Chi-square test as described before.

Fig 4.4 Different forms of *B.bovis* parasites in culture.

Fig 4.4



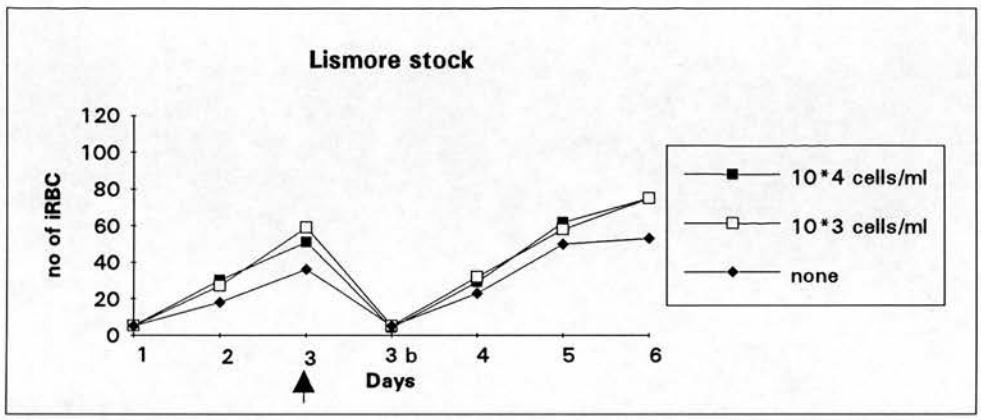
4.3.2.1 Results

The medians of parasite counts obtained in smears from wells with MPWC were significantly higher ($p< 0.001$) than the control wells for all three stocks of *B.bovis* and no significant differences ($p> 0.05$) were found between the wells which had received 10^4 or 10^3 MPWC/ml in any of the stocks. The counts for each well are presented in Appendix 2 and the medians of replicates are presented in Fig 4.5 A-C.

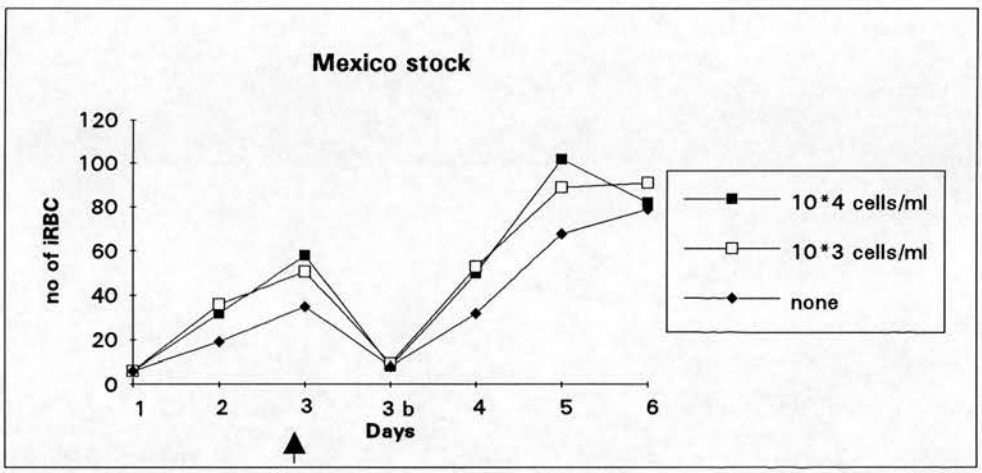
Throughout the morphological analysis, no triplet forms were seen in any of the cultures and quadruplet forms, when seen, were very few representing a maximum of 1% of the total counts. Thus, the counts of these two forms were not included in the figures. The culture wells of Lismore and Mexico stocks which had received MPWC at a concentration of 10^4 cells/ml contained significantly fewer ($p< 0.001$) pycnotic forms of *B.bovis* than the control wells as shown in Fig 4.6A and 4.7A. With the Mexico stock there were also a significant increase ($p< 0.001$) in the number of pair and free forms and a decrease in the number of single forms in the wells with 10^4 MPWC/ml when compared with the control wells. There were no significant differences between counts of single forms in any of the MPWC treatments or in control cultures (Fig 4.6 A-C and Fig 4.7 A-C). The morphological analysis of Kwanyanga stock (Fig 4.8 A-C) showed no significant difference ($p> 0.05$) between the counts of the different parasite forms observed from wells with MPWC either at 10^4 or 10^3 cells/ml and the control wells. The counts of the different forms obtained from each stock of *B.bovis* are presented in Appendix 2.

Fig 4.5 Number of *B.bovis* infected cells in 1,000 RBC of established cultures of Lismore (A), Mexico (B) and Kwanyanga (C) stocks with MPWC as feeder cells. Day 3b represents counts on day 3, after subculture.

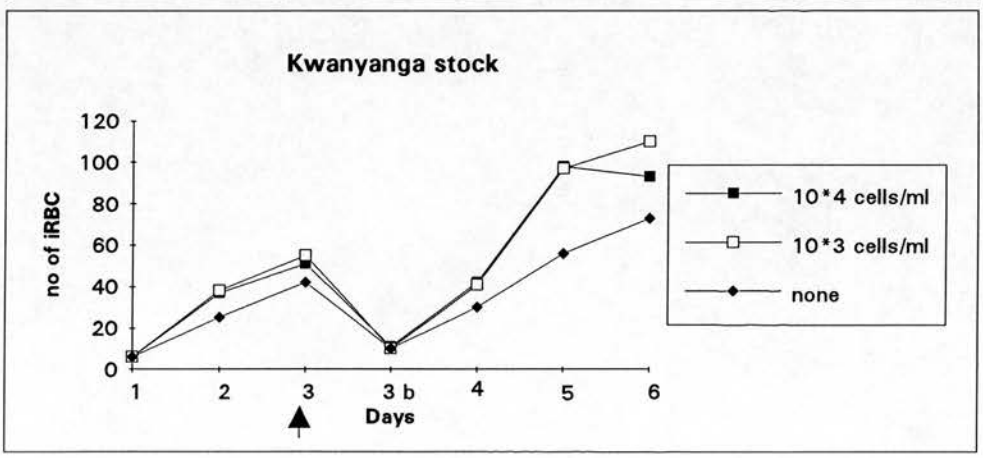
Fig **4.5**


Lismore stock
no of iRBC
120
100
80
60
40
20
0
1
2
3
3 b
4
5
6
Days
10*4 cells/ml
10*3 cells/ml
none

A


Mexico stock
no of iRBC
120
100
80
60
40
20
0
1
2
3
3 b
4
5
6
Days
10*4 cells/ml
10*3 cells/ml
none

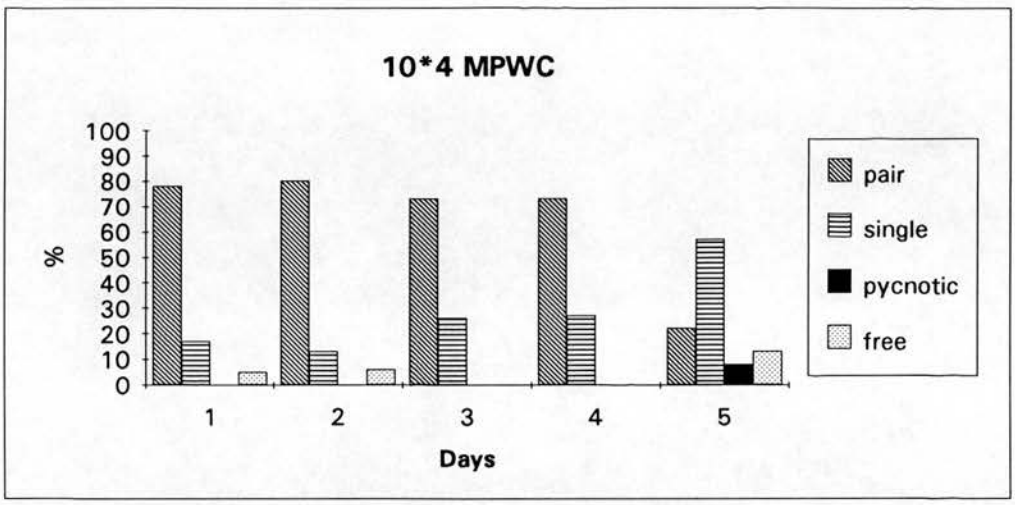
B


Kwanyanga stock
no of iRBC
120
100
80
60
40
20
0
1
2
3
3 b
4
5
6
Days
10*4 cells/ml
10*3 cells/ml
none

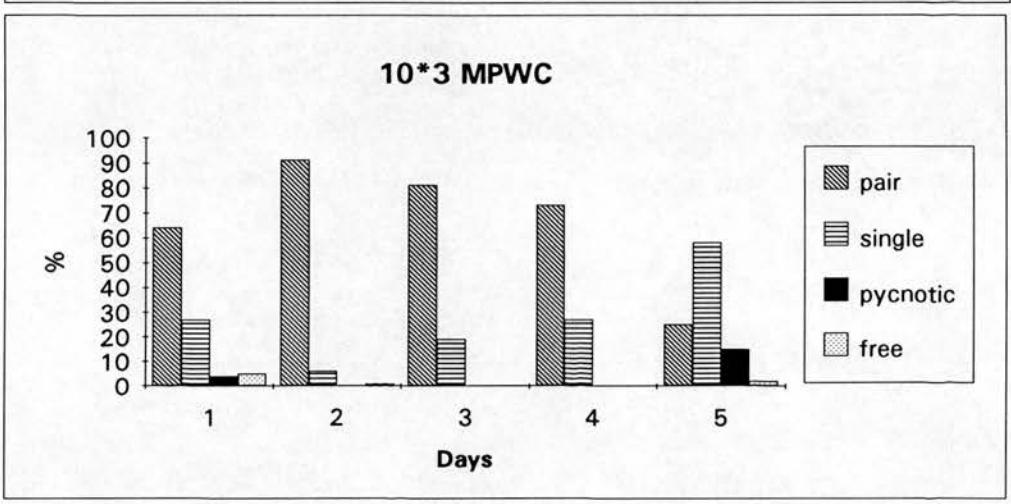
C

Fig 4.6 Proportions of different forms of *B.bovis* parasites (Lismore stock) in cultures with MPWC at a concentration of 10^4 cells/ml (A), 10^3 cells/ml (B) and in control cultures (C).

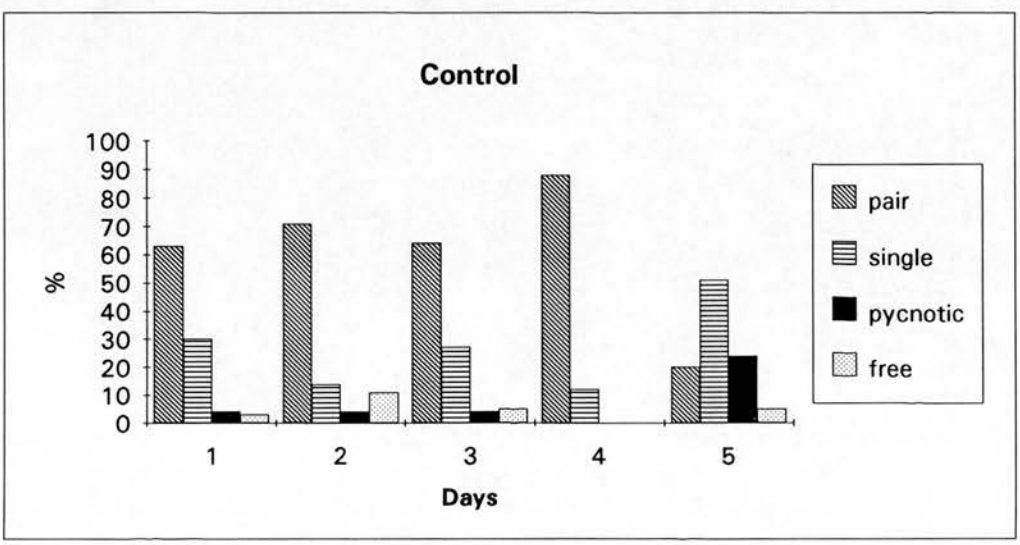
Fig 4.6


10*4 MPWC
%
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Days
pair
single
pycnotic
free

A


10*3 MPWC
%
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Days
pair
single
pycnotic
free

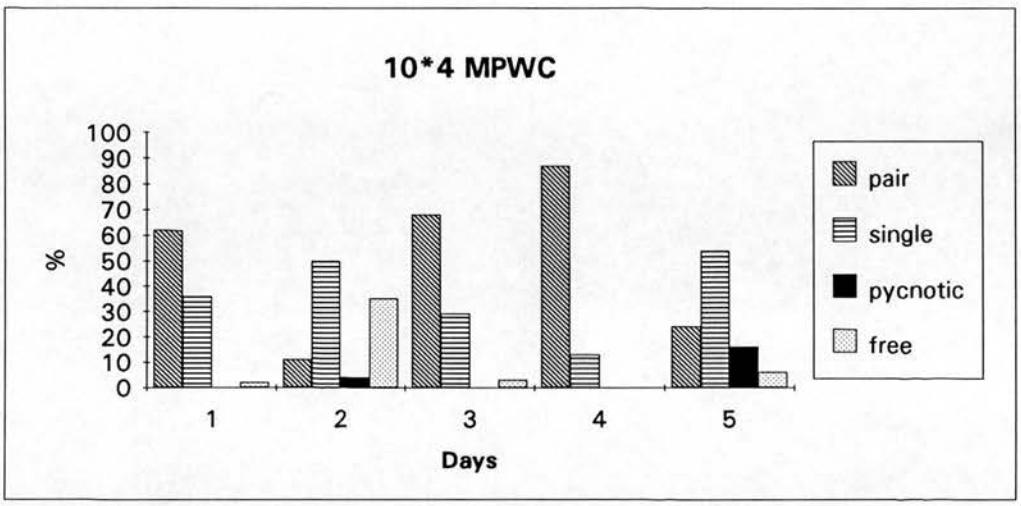
B


Control
%
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
Days
pair
single
pycnotic
free

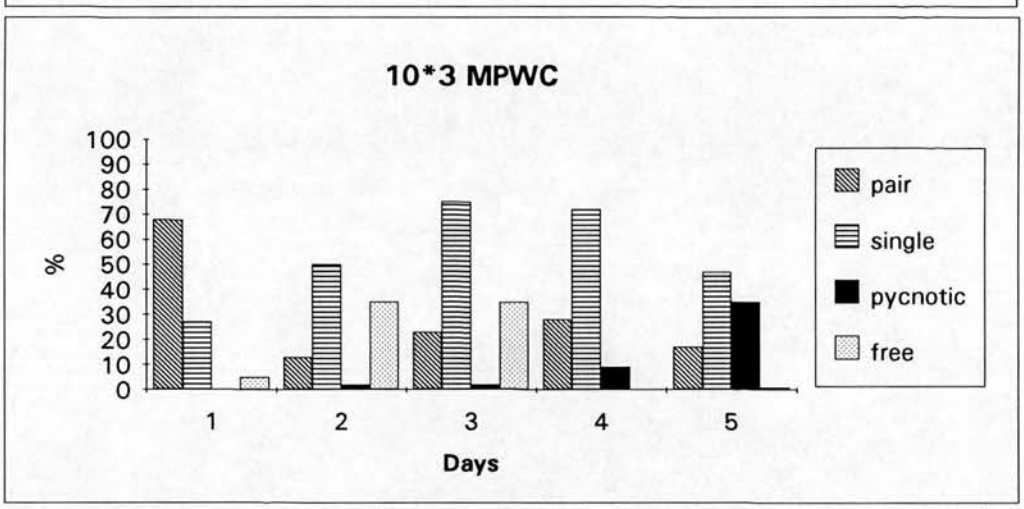
C

Fig 4.7 Proportions of different forms of *B.bovis* parasites (Mexico stock) in cultures with MPWC at a concentration of 10^4 cells/ml (A), 10^3 cells/ml and in control cultures (C).

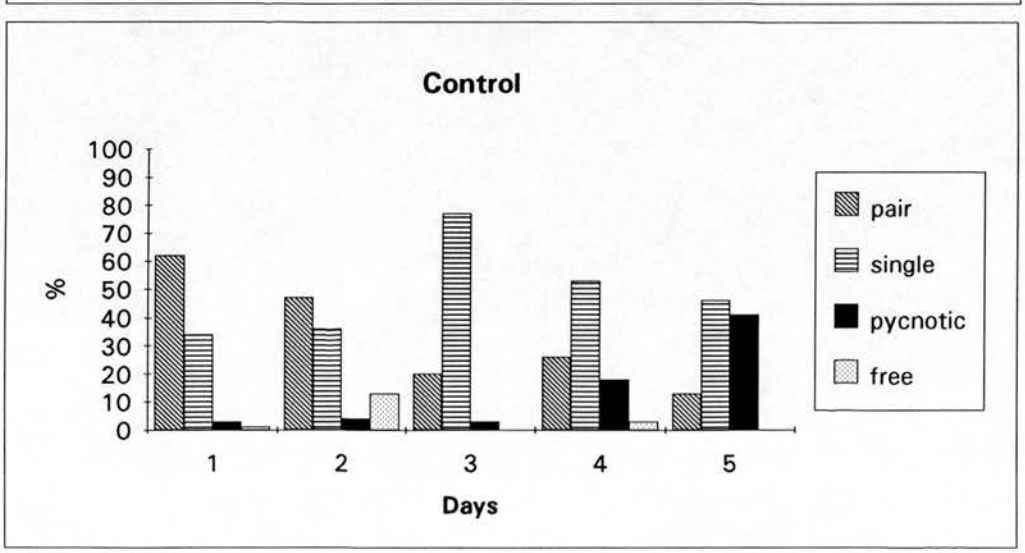
Fig 4.7


10*4 MPWC
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

A


10*3 MPWC
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

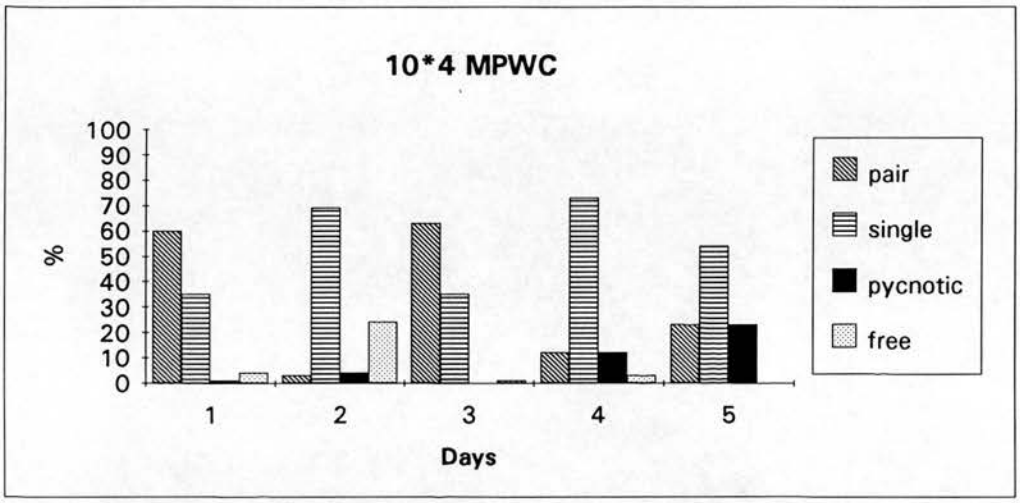
B


Control
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

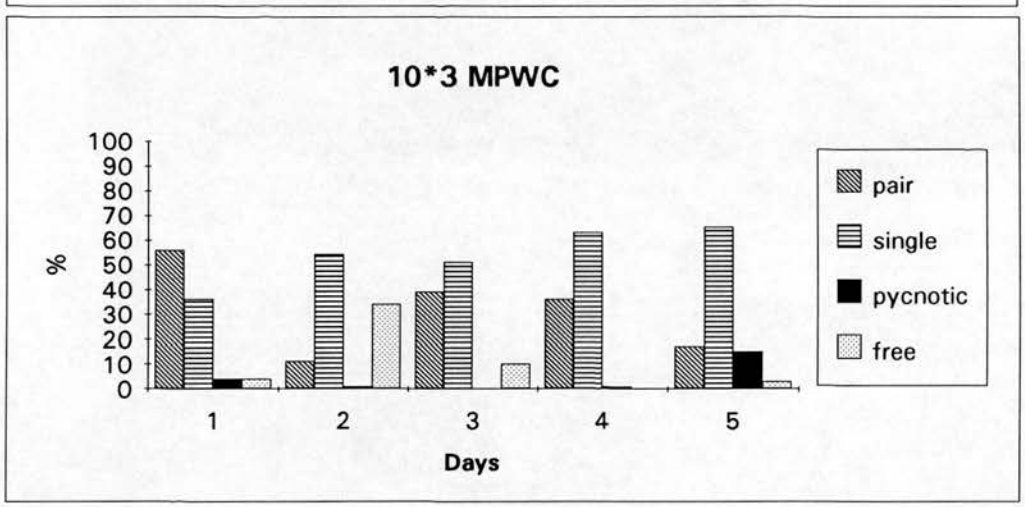
C

Fig 4.8 Proportions of different forms of *B.bovis* parasites (Kwanyanga stock) in cultures with MPWC at a concentration of 10^4 cells/ml (A), 10^3 cells/ml and in control cultures (C).

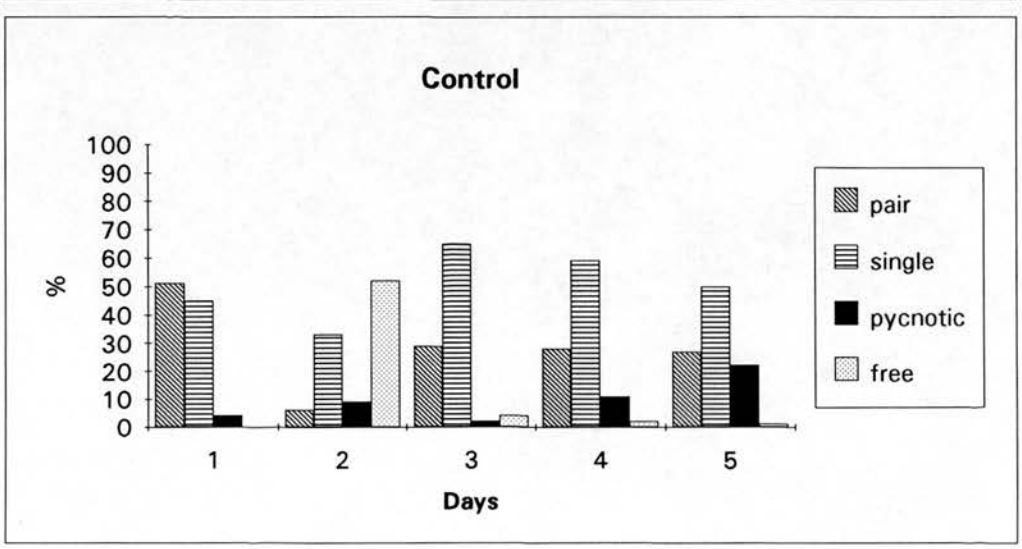
Fig **4.8**


10*4 MPWC
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

A


10*3 MPWC
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

B


Control
100
90
80
70
60
50
40
30
20
10
0
%
1
2
3
4
5
Days
pair
single
pycnotic
free

C

4.3.3 Effect of MPWC on resuscitation

Due to the fact that no significant differences on parasite counts were observed between 10^4 and 10^3 MPWC/ml for established cultures, MPWC were tested only at a concentration of 10^4 for *in vitro* resuscitation of each stock of *B.bovis*.

On day -1, four wells of three 24-well plates (one for each stock) received 1 ml of a 10^4 MPWC suspension, which had been harvested and prepared as described before, and four wells received culture medium (control wells).

On day 0, for each stock, two vials of *B.bovis* cryopreserved cultured parasites (see section 3.3.4) were resuscitated as described in section 3.3.5, pooled, and 1.25 ml aliquots of the resultant 10% RBC suspension were used to inoculate each well. The plates were incubated at 37°C under reduced O_2 tension. Medium was changed and cytospin smears were made from each well daily until day 3. Six fields of approximately 750 RBC each (4,500 RBC in total) were examined and the number of infected erythrocytes recorded.

The medians of parasite counts from each set of replicates were compared by the Chi-square test, as described before.

4.3.3.1 Results

No significant differences ($p > 0.05$) were observed between the medians of parasite counts from the wells with MPWC and the medians of counts from the control wells in any of the stocks tested. The counts obtained from each well are presented in Appendix 2.

4.3.4 Discussion

In the present study, the BAE cells did not affect parasite growth, whereas the MPWC, used at concentrations of 10^4 or 10^3 cells/ml, were shown to support an increase in the parasitaemias of established cultures of all three stocks of *B.bovis* tested. These findings suggest that the MPWC might be useful in increasing parasitaemias of *B.bovis* isolates that are more difficult to growth *in vitro*. There is also a possibility that the MPWC might be helpful in establishment *in vitro* of sub-populations which are more difficult to grow, which may be an important consideration in cloning experiments.

An increased multiplication rate of *P.falciparum* has also been reported when MPWC were incorporated into cultures (Trenholme & Phillips, 1989). However the mechanism by which

the MPWC promote this is not completely clear. It is suggested that either the cells release a labile cysteine that acts as a promoting factor or that the adherent cells, which are mainly macrophages, might remove toxic metabolites which inhibit parasite growth. It is possible that the MPWC, as suggested for *P.falciparum*, had a positive effect on *B.bovis* growth by releasing promoting factors into the culture medium or by removing from the medium toxic metabolic products generated during parasite multiplication.

Further evidence of an effect of macrophages on growth of *Babesia* parasites was given by Tambrallo and co-workers (Tambrallo et al, 1992), who reported increase of *in vitro* growth of *B.bovis* and *B.bigemina* when a bovine recombinant granulocyte macrophage/colony stimulating factor was incorporated into cultures (Tambrallo et al, 1992). However the mechanism by which this stimulating factor affected the parasite growth was not investigated. The same factor also enhanced promastigote growth of *Leishmania mexicana amazonensis* in mice and this was proposed that it was due to a shortening of the parasite cell cycle (Charlab et al, 1990).

Regarding the morphological analysis, there was great variation in the results, and it was difficult to draw substantial conclusions except that the incorporation of MPWC at 10^4 cells/ml in established cultures resulted in significantly fewer pycnotic forms of Lismore and Mexico parasites.

Pycnotic forms are usually a sign of unhealthy cultures and commonly occur as a result of inadequate culture conditions, such as delay in medium change, delay in subculture or low concentration of O_2. The presence of pycnotic forms is therefore undesirable and in general means that the culture conditions are not optimum. The presence of fewer pycnotic parasites in cultures with MPWC gives support to the hypothesis that mouse macrophages may produce better culture conditions by removing toxic metabolites from the culture medium.

The wide variation in the distribution of different forms observed throughout the examination period can possibly be explained by the cultures being in different stages of development at the moment of sampling. On day 2 for instance, a high proportion of free merozoites was found particularly in Mexico and Kwanyanga stocks in all culture conditions; it may be because at that particular moment many merozoites had been released into the culture medium and were about to start another cycle by infecting new RBC.

In general it was clear that the Lismore stock showed consistently more pair forms and fewer free merozoites than the other two stocks, independently of the use of MPWC. On the other hand, parasitaemias of Mexico and Kwanyanga stock cultures were always higher than those of Lismore stock.

Although the MPWC promoted increases in parasitaemias of established cultures, a concentration of 10^4 MPWC/ml did not promote increase in parasitaemias when tested in *in vitro* resuscitation of any of the stocks tested. Perhaps the MPWC did not produce any effect on resuscitation because the conditions used on this occasion were quite adequate in terms of RBC and serum supplies. This theory was supported by the finding of parasitaemias around 4% on day 3 after resuscitation, even in cultures without MPWC, which is higher than the ones routinely obtained at this point during previous resuscitations at CTVM, which have been 1% or less. Besides, it has to be considered that, although on some occasions stabilates of Kwanyanga and Mexico stocks have been difficult to resuscitate (L.Bell-Sakyi, personal communication), the three isolates used in this experiment were originally established *in vitro* from infected blood quite easily, and have been cultured *in vitro* on a number of subsequent occasions. Although the MPWC did not seem to improve the resuscitation of stabilates of *B.bovis* stocks used in this experiment, they may be useful in establishment of new isolates which have never been cultured *in vitro*.

4.4 CLONING OF *B.BOVIS*

Previous work carried out at CTVM on glucose phosphate isomerase (GPI) isoenzyme analysis by gel electrophoresis of stocks of *B.bovis* found that the Lismore stock of *B.bovis*, which was originally a virulent strain isolated from a clinical case (Kahl et al, 1983), showed two bands of this enzyme, whereas the Kwanyanga and Mexico stocks had only one band (T.R.Melrose, personal communication). The GPI pattern has shown to be useful as a biochemical marker for characterisation of different subpopulations of *Theileria* parasites (Melrose & Brown, 1979). Furthermore, analysis of *B.bovis* parasites at both the protein and DNA levels has demonstrated that a high proportion of the isolates are composed of a heterogeneous mixture of subpopulations (Kahl et al, 1982b; 1983; Cowman et al, 1984). Therefore, at the beginning of this study, it was believed that the Lismore stock of *B.bovis* might be composed of heterogeneous populations of parasites differing in antigenicity and consequently complicating the immunochemical characterisation (described in Chapters 6 and 7). An *in vitro* culture procedure has been developed for cloning and establishment of homogeneous populations of *B.bovis* parasites (Rodriguez et al, 1983).

Thus, a single experiment was designed with the aim of cloning the Lismore isolate by limiting dilution in order to obtain cloned lines for immunological characterisation.

The experiment was designed to clone cultured parasites by limiting dilution *in vitro* in a first stage, and then to reclone the resultant lines to ensure their origin from a single parasite. For comparative analysis of parent and cloned lines, three parameters were used: evaluation of growth rates *in vitro*, morphology of the parasites and isoenzyme (GPI) profiles.

4.4.1 Cloning procedure

A vial of culture stabilate of the Lismore stock of *B.bovis,* passage 11, was resuscitated into a 25 cm^2 flask (as described in section 3.3.5) and was cultured continuously *in vitro* for 1 week, during which time it was subcultured twice. The cloning procedure was carried out by limiting dilution according to Rodriguez and co-workers (Rodriguez et al, 1983) with some modifications.

The number of RBC per µl in a sample of the culture suspension, counted in an electronic particle counter, was 1.49 x 10^6 and the parasitaemia estimated in a Giemsa-stained thin smear (see section 3.2.3), was 2.53%. Thus, 26 µl of culture suspension were mixed with 174 µl of M199-H in order to give 10^6 iRBC/200 µl, which was used to make serial dilutions. Serial 10-fold dilutions were then made in a 96-well plate (parent culture wells) to result in a

final dilution of 200 µl that contained one infected RBC. This final dilution was divided into four aliquots which were put into individual wells in a separate 96-well plate and constituted a cloning set. The same procedure was carried out six times resulting in six cloning sets in separate plates. To give a final depth of 4.0 mm (total volume of 180 µl/well), 130 µl of 10% RBC suspension (as described in 3.3.1) was added to each well. The parent culture wells were kept as controls. The plates were incubated under reduced O_2 atmosphere. The medium in each well was changed daily by removal and replacement of 100 µl of the overlying medium.

The control wells were examined on day 3 by making cytocentrifuge smears (see section 3.3.3). On day 9 cytospin smears from the cloning sets were made and examined. The contents of any cloning well showing parasites were transferred to wells of 24-well plates and 1 ml of 10% RBC suspension was added to each well. The plates were cultured for two days and the contents of each well was transferred to 25 cm^2 flasks; the volume in each flask was adjusted to 5 ml with 10% RBC suspension. The cloned lines were cultured in flasks for two days and were cryopreserved in 5% DMSO as described in 3.3.4. On days 12 and 15 the remaining negative cloning wells were screened again in order to detect possible slow-growing clones.

4.4.1.1 Results

From the 24 cloning set wells (6 cloning sets with 4 wells each) in the 96-well plate, 17 showed growth and 13 were transferred to individual wells in a 24-well plate, as described in Table 4.1.

Screening of negative wells from the cloning sets on days 12 and 15 did not detect any slow-growing clone.

The contents of six wells from the 24-well plate (originally well 1 of cloning set 1, well 2 of cloning set 2, well 4 of cloning set 3, well 1 of cloning set 4, well 4 of cloning set 5 and well 2 of cloning set 6) were transferred to six individual 25 cm^2 flasks, which were designated clones 1, 2, 3, 4, 5 and 6 respectively.

Table 4.1 *In vitro* growth of *B.bovis* cloning sets

	Well No			
cloning set No	1	2	3	4
1	++++ *	+++	+++	+++
2	++ *	+++ *	--	--
3	--	--	+	++ *
4	++ *	++ *	+++ *	++ *
5	--	--	++ *	++ *
6	++ *	+++ *	--	++ *

Key: -- no parasites seen
++ less than 2 parasites/field
+++ 2 to 4 parasites seen/field
++++ more than 6 parasites seen/field

* contents transferred to 24-well plate wells

4.4.2 Comparison of cloned lines

Stabilates of the parent line and of the clones 1, 3 and 4 were resuscitated into 25 cm^2 flasks, 3 weeks after cryopreservation, and were compared by growth rate, morphology and isoenzyme profiles.

4.4.2.1 Growth rate

The growth rate was evaluated by culturing the parent line and the cloned lines in 24-well plates, starting from three different parasitaemias (1%, 0.1% and 0.01%), each with four replicates. There was no subculturing but the medium was changed daily. The parasitaemia was determined daily for 7 days by counting 2,000 RBC in Giemsa stained smears made from each replicate well. For parasitaemias below 0.5% at least 100 fields (about 250 cells/field) were counted.

4.4.2.2 Morphological analysis

On day 7, smears from the replicates which had started with 0.01% of parasitaemia were used for morphological studies. At least 100 parasites were counted in each slide and were classified according to their morphology as: single, pair, quadruplet, pycnotic and free parasites (as described in Fig 4.4). Data obtained from growth rate evaluation and morphological studies were statistically analysed using the Chi-square test for homogeneous frequencies (Fowler & Cohen, 1992).

4.4.2.3 GPI analysis

The GPI analysis was done in collaboration with Mr T.R.Melrose, CTVM. Samples for isoenzyme study of parent and cloned lines were prepared by selective lysis using KCl as described in section 3.6.1.1 and frozen at -80^{o}C as a pellet. The same procedure was carried out for uninfected RBC suspension as a negative control.

Electrophoresis was carried out according to Melrose & Brown (1979). Briefly the parasites were lysed by freeze-thawing twice and the lysates were inserted on pieces of washed white embroidery cotton in a plate with a layer of starch gel 0.9 mm thick. Electrophoresis was carried out for 3 hours at 8^{o} C with a voltage of 350 V and current of 25 mA. An electron transfere dye system was used to locate the bands of GPI isoenzyme activity. The developer was prepared by dissolving the substrate, the auxiliary enzyme glucose-6-phosphate

dehydrogenase and the co-enzyme in a 0.272 M potassium phosphate buffer (pH 7.0). Approximately 100 mg purified agar was dissolved in 10 ml distilled water by heating in a boiling water bath, then cooled to 50° C and added to the developer solution. The agar overlay was poured into a plastic mould resting on top of the gel and the complete plate tilted to spread the solution over the whole area of the plate. The plate was incubated at 37° C for 1 hour and the developed gel was photographed as described in Appendix 3.

4.4.2.4 Results

The medians of parasite counts in replicate cultures of the parent and the three cloned lines from starting parasitaemias of 1%, 0.1% and 0.01% are presented in Fig 4.9 (A), (B) and (C) respectively.

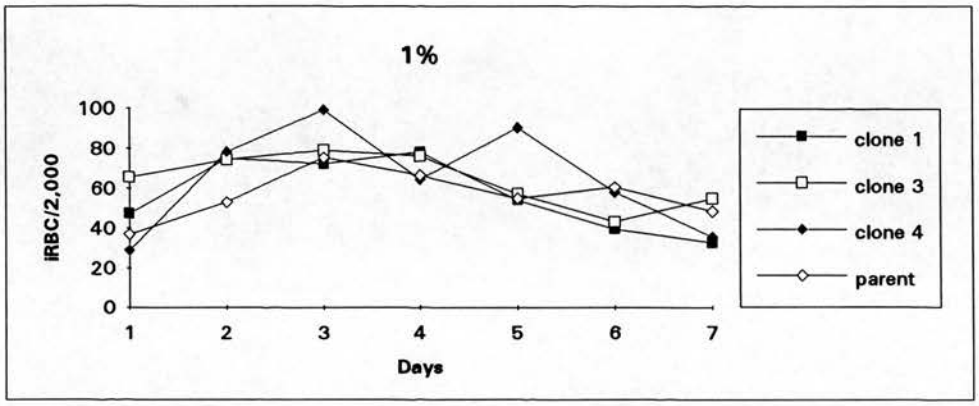
There were no significant differences ($p > 0.05$) between the medians from the parent and any of the cloned lines in cultures initiated with parasitaemias of 1% or 0.1%. However, at a starting parasitaemia of 0.01%, clone 4 showed significantly lower parasite counts ($p < 0.001$) when compared with counts of the parent and clones 1 and 3 cultures (Fig 4.9 C).

No significant differences between the cultures (parent, clone 1, 3 or 4) ($p > 0.05$) were found in proportions of any of the parasite forms. The proportions of different parasite forms in cultures of parent and cloned lines are presented in Fig 4.10.

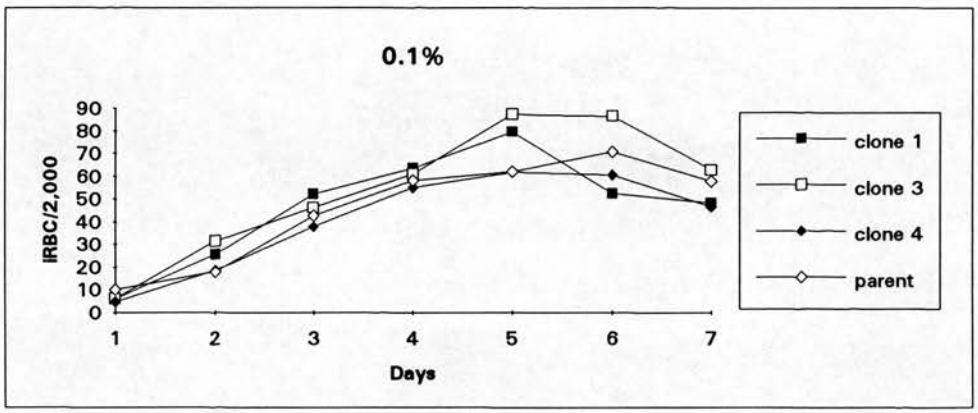
The GPI isoenzyme banding patterns are shown in Fig 4.11. The uninfected RBC showed two cathodic bands of activity. All *B.bovis* samples (parent and cloned lines) showed a single anodic band with the same pattern of migration in every sample; however it was demonstrated that the GPI bands observed were of *B.bovis* origin, since they were absent in the uninfected RBC control.

Fig 4.9 *In vitro* growth of clone 1 (--■--), clone 3 (--□--), clone 4 (--◆--) and parent line (--◊--) of *B.bovis* (Lismore) starting from a parasitaemia of 1% (A), 0.1% (B) or 0.01% (C) over a period of 7 days in culture.

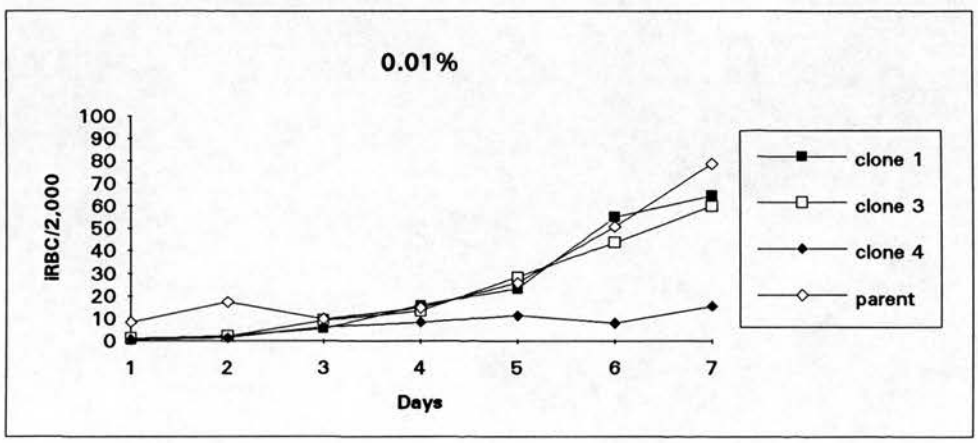
Fig 4.9


1%
iRBC/2,000
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
clone 1
clone 3
clone 4
parent

A


0.1%
iRBC/2,000
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Days
clone 1
clone 3
clone 4
parent

B


0.01%
iRBC/2,000
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Days
clone 1
clone 3
clone 4
parent

C

Fig 4.10 Proportions of different forms of *B.bovis* parasites in cultures of parent line (▧), clone 1 (▤), clone 3 (▩) and clone 4 (▥).

Fig **4.10**

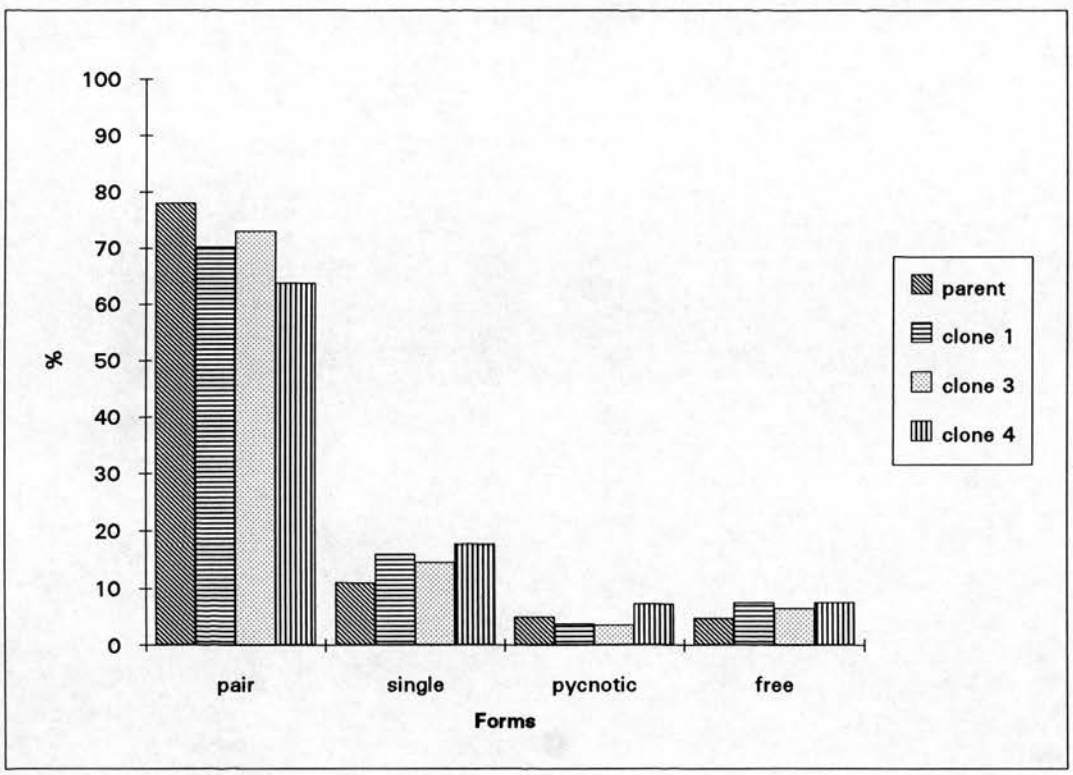

100
90
80
70
60
50
40
30
20
10
0
%
pair
single
pycnotic
free
Forms
parent
clone 1
clone 3
clone 4

Fig 4.11 Zymogram of a parent and three cloned lines of *B.bovis* (Lismore) stained for GPI activity.

Lysates prepared from:

Clone 1 (lane a)

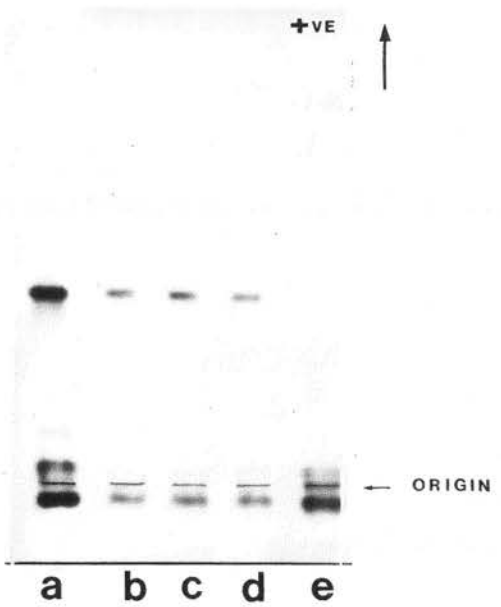
Clone 2 (lane b)

Clone 4 (lane c)

Parent line (lane d)

Lane e is an uninfected RBC control.

Fig 4.11



4.4.3 Recloning procedure

The comparative study of the parent and cloned lines using three parameters (growth rate, morphology and GPI patterns) showed that all lines were very similar. However, when cultures were initiated from low parasitaemia (0.01%), for evaluation of growth rate, a significant difference was detected between parasitaemias of clone 4 and the other cultures. Therefore, clone 4 was selected for recloning.

A culture stabilate of clone 4 was resuscitated *in vitro* (section 3.3.5) and cultured for 1 week in two 25 cm^2 flasks, which provided culture suspensions for a recloning experiment.

The number of RBC per µl of culture suspension from the two flasks was 1.45×10^6 and 1.53×10^6, and parasitaemias were 4.8% and 4.6% respectively. Therefore, one µl of each culture suspension contained approximately 70,000 iRBC.

The recloning procedure was based on the method described by Rosario (1981). The change in methodology was due to the high number of positive wells obtained in the first cloning sets (see 4.4.1.1).

The culture suspensions were diluted with M199-H to give a final concentration of 0.25 iRBC/µl and a PCV of 2% as described:

Dilution			**Parasite concentration**
1	10 µl of culture	690 µl of medium	10^5 iRBC/100 µl
2	10 µl of dilution 1	990 µl of medium	10^3 iRBC/100 µl
3	100 µl of dilution 2	900 µl of medium	100 iRBC/100 µl
4	100 µl of dilution 3	900 µl of 2% RBC suspension	10 iRBC/100 µl
5	200 µl of dilution 4	1.8 ml of 2% RBC suspension	1 iRBC/100 µl
6*	1.5 ml of dilution 5	4.5 ml of 2% RBC suspension	0.25 iRBC/100 µl

* used to initiate 2 cloning sets (50 wells in each of two 96-well plates)

One hundred µl of the dilution 3 were placed into each of 5 wells in a 96-well plate and were designated control A (100 parasites). The same procedure was done with dilution 4 and the wells were designated control B (10 parasites).

One hundred µl of the dilution 6 (0.25 parasite) were then placed into each of 50 wells of two 96-well plates (cloning sets). Thus, parasite growth was expected in 12.5 wells of each plate.

Thirty µl of a 2% RBC suspension were added to each well (including control A and B wells) and the plates were incubated under 4% O_2 gas tension. Medium was changed in the control wells on days 1, 3, 5 and 7. On days 2, 4, 6 and 8 100 µl of overlying medium of each control well was replaced by 100 µl of a 2% RBC suspension, resulting in a PCV of 10% in all wells on day 8. Each day, starting from day 6, a different well of each control set (A and B) was screened by preparing and examining cytocentrifuge smears. The cloning set wells also had the overlying medium (100 µl) replaced by 100 µl of a 2% RBC suspension on days 2, 4, 6 and 8, resulting in a PCV of 10% on day 8. From then, the overlying medium (100 µl) was changed daily in the control wells and every 2 days in the cloning set wells until day 15, when cytospin smears were made from each of the cloning set wells.

Cloning set wells showing parasites were transferred to individual wells of a 24-well plate and 1.2 ml of 5% RBC suspension were added to each well. The plate was incubated under 5% CO_2 in air and medium was changed 24 hours later. After 48 hours of incubation, 1 ml of the overlying medium in each well was replaced by 1 ml of a 5% RBC suspension and thereafter the medium (1 ml) was changed daily.

The 25 cm^2 flasks which had been used to provide the original clone 4 cultures for initiation of the cloning set wells were kept as control under standard conditions with daily medium changes and subcultures every 2 days.

4.4.3.1 Results

Six wells in one plate and 8 in the other were positive on day 15 and were transferred to 14 individual wells of a 24-well plate. On day 20, a further 5 wells in the first plate were positive and were transferred to the 24-well plate; no more positive wells were detected in the second plate. On day 21, the first transferred wells in the 24-well plate were examined again and in all of them very few of the parasites seen were healthy. None of the parasites in the 24 well plate or in the control wells (A and B) in the 96-well plate survived beyond day 24 and therefore no cloned lines were obtained after recloning.

The original clone 4 cultures (the two 25 cm^2 flasks) used to initiate the cloning sets were still healthy and attaining parasitaemias of around 5% 48 hours after subcultures.

4.4.4 Discussion

The results obtained from the first cloning procedure used in this study indicated that the culture conditions were adequate and allowed the establishment of cultures from a very low

starting parasitaemia. However, at the end of the cloning procedure, the number of cloned lines obtained was higher than expected (Table 4.3). All of the six cloning sets, which theoretically had been initiated with 0.25 parasite/well and should have resulted in growth in one single well, resulted in growth in more than one well. In two cases (cloning sets 1 and 4) all four wells showed parasite growth, proving that the starting concentration of parasites was at least 1 parasite/well. These results may be due to several factors. Firstly, due to a possible inaccuracy of parasitaemia estimations in Giemsa stained smears; secondly, due to an inaccuracy of the serial dilutions needed to obtain 1 parasite/µl, which involved six dilution steps consequently increasing the chance of errors; thirdly, it is possible that by the time the cloning procedure actually finished, the number of parasites/µl of culture suspension was higher than when the cultures had been sampled. The period of time between the sampling and the actual completion of the cloning procedure (including time required for counts of RBC and parasitaemia, carrying out dilutions, etc.) was approximately $1^{1/2}$ hours which may permit substantial changes on the status of a *B.bovis* culture, in which doubling time in an exponential growth phase is between 7 and 10 hours (Kellermann et al, 1988). In addition, absence of CO_2 induces release of free merozoites into the culture supernatant; the period of time that the cultures were being manipulated in the microflow hood, deprived of CO_2, before the cloning wells were set up, may have resulted in release of some of the pair forms of *B.bovis* parasites as 2 free merozoites in the culture supernatant and therefore in an increase of the number of infective forms. One possible way to diminish the error in estimation of starting number of infective parasites would be by washing the cells with medium before initiation of the cloning procedure and consequently eliminating most of the free merozoites.

Although the number of parasite lines obtained after cloning was higher than expected and the culture lines obtained did not seem to be established from a single parasite, they were derived from very few parasites and could each represent a more homogeneous population than the original Lismore stock, and therefore be useful for comparative studies of immunochemical characteristics.

From the growth rate analysis, clones 1, 3 and 4 showed exponential growth very similar to that of the parent line in cultures initiated with 0.1% or 1% parasitaemia (Fig 4.9 B and 4.9 C). When a starting parasitaemia of 0.01% was tested, clones 1 and 3 had growth rates again very similar to that of the parent line; in contrast, clone 4 appeared to be a much slower growing culture when compared with the parent and the other two lines (Fig 4.9 A). For this reason, clone 4 was chosen to be recloned in a second experiment, in which some modifications were introduced with the aim of improving the efficacy of the cloning procedure.

The morphological studies of the different culture lines did not show remarkable differences in proportions of the different forms of the parasite after 7 days in culture.

The results obtained from the GPI analysis are in agreement with observations reported by Rodriguez and co-workers, who described the presence of a single anodic band of GPI, and also the presence of lactate dehydrogenase, glutamate dehydrogenase and hexokinase in four Mexican isolates of *B.bovis*, including one cloned line (Rodriguez et al, 1986b). In that study no variation was detected between the isolates with any of the enzymes tested. The presence of GPI in *Babesia* parasites is expected as previous reports indicate that glycolysis is the major pathway of energy production in *B.rodhaini* (Rickard, 1970). Similar results have been reported for *B.bigemina* parasites by Vega and co-workers, who found an identical single anodic band present in the parent and in three cloned lines of a Mexican isolate (Vega et al, 1986b).

The analysis of isoenzymes has been reported to be a useful tool in discrimination of subpopulations of other parasites such as *Plasmodium* (Graves et al, 1984) and *Theileria* (Melrose & Brown, 1979), which have shown polymorphism of the parasite-specific enzymes. However it does not seem to be the case with *Babesia*, since polymorphism has not been observed amongst isolates of *B.bovis,* which were expected to be composed of different subpopulations as a consequence of passages through splenectomised calves, serial passages in cultures or cloning by serial dilution (Rodriguez et al, 1986b). The lack of polymorphism has also been reported for cloned lines of *B.bigemina* (Vega et al, 1986b) and different isolates of *B.microti* (Momen et al, 1979). Therefore, the fact that all the lines of *B.bovis* analysed in this study showed the same GPI pattern does not necessarily mean that they are identical populations; it may be the case that the analysis of enzymatic contents is not a suitable marker to identify polymorphism of subpopulations in *Babesia* parasites. On the other hand, enzymatic characterisation can be very useful in taxonomic studies, especially in discrimination between morphologically similar species, since inter-species enzymatic variation between *B.bovis* and *B.bigemina* has been reported (Rodriguez et al, 1986b; Vega et al, 1986b).

The method by which clone 4 was recloned appeared to be more accurate than the first cloning method used, hence the numbers of positive wells obtained after cloning were similar to that expected. Amongst the 50 wells inoculated with 0.25 parasites, one would expect to have parasite growth in 12.5 of them; the results obtained after recloning showed 11 wells in one plate and 8 wells in the second plate having parasite growth. Nevertheless, none of the clones obtained after recloning of clone 4 and none of control wells survived after 24 days in culture.

The ability of *B.bovis* parasites (clone 4) which had been kept in the original 25 cm^2 flasks to survive and multiply beyond 24 days in culture, associated with the fact that the control wells (A and B) in the 96-well plates did not survive, suggests that the culture conditions of the recloning set wells were not appropriate to maintain established cultures continuously *in vitro*. The failure to keep those recloned lines as continuously growing cultures was probably due to the fact that they had been under reduced O_2 tension for a very long time, rather than to unsuitability of RBC and serum supplies, which were the same used to feed the cultures in the flasks. The cloning set wells in 96-well plates were incubated under reduced O_2 tension for 15 days before being screened, and only at that time were the contents of the first positive wells transferred to 24-well plates and incubated under 5% CO_2 in air, which is the standard gas tension for cultures in the exponential phase of growth. The second detection of clones was carried out 20 days after the cloning set wells had been initiated and therefore the clones detected at this time had been under reduced O_2 gas tension for 20 days. Such a long period of reduced O_2 incubation may have resulted in cultures with less healthy parasites, which were unable to multiply continuously *in vitro*. From other experiments carried out at CTVM using *in vitro* cultures of *B.bovis*, and from published data (Rodriguez et al, 1983), there is no doubt that the use of a reduced O_2 atmosphere is essential for initiation of cultures from a low parasitaemia (particularly in resuscitation of frozen stabilates); however it is also clear that as soon as the parasites have become established as a growing culture it is essential to change the incubation atmosphere to 5% CO_2 in air. This fact has been observed especially with Lismore stock, which is the easiest isolate of *B.bovis* to re-establish as a growing culture from frozen stabilates; it requires a shorter period of incubation under reduced O_2 tension, and can if necessary be resuscitated straight into 5% CO_2 in air (L.Bell-Sakyi, personal communication).

Two other parameters of the culture system used for the recloning were different from the conditions used for cultures in flasks: the PCV and the medium depth. The PCV of cultures in 96-well plates was initially 2% and was increased gradually achieving the standard value of 10% only 8 days after initiation of cultures, whereas the PCV of cultures in the flasks was always maintained at 10%. The effect of different concentrations of RBC suspensions on *B.bovis* growth *in vitro* has been studied by Goff and Yunker (1986), who reported no significant differences in growth rates due to variation of PCV; in addition, an increase of the percentage of iRBC was observed when the PCV of cultures was reduced sequentially during exponential growth even though the total number of parasites per culture remained the same (Goff & Yunker, 1986). However in that study, the reduction of the PCV was evaluated using a growing culture with initial PCV of 10% and a parasitaemia of 1.5%, contrary to the present study, in which cultures had to be initiated from much lower parasitaemia using a low initial PCV (2%). The lack of *in vitro* development of *B.bigemina* parasites when a PCV of

2.5% was used has been reported by Vega and co-workers, who found that the optimum PCV was between 5 and 10%, and suggested that a strong contact or pressure between the RBC is required for *B.bigemina* to penetrate new RBC (Vega et al, 1986a). That study also suggests that variation of PCV affects development particularly of cultures with low number of parasites (parasitaemia < 0.1%). This also might be the case for *B.bovis* parasites. The depth of the medium in 96-well plates was 4 mm (130 µl/well), which according to Rodriguez and co-workers is the best fluid level to initiate cultures from a low parasitaemia (Rodriguez et al, 1983), whereas the depth of the medium in the flasks was around 7 mm (5 ml/ flask), which is the fluid level routinely used for cultivation of all three stocks of *B.bovis* at CTVM. The reduction of the PCV and medium depth in the 96-well plates could also have contributed to the failure in maintenance of recloned lines and control wells continuously *in vitro.*

An analysis of the influence of different environmental conditions on genetically distinct subpopulations of *B.bovis* has been published recently in which several samples were characterised using a recombinant DNA probe specific for a tandemly repeated sequence located within the BoVAI gene (Dalrymple et al, 1992). In that study it was concluded that most samples derived from single animals contained more than one genetically distinct population, although the attenuated strain (Ka), which had been used as a live vaccine for 11 years in Australia, contained only one parasite population. On the other hand the replacement attenuated vaccine line (Ta), which is in use nowadays, contained two distinct subpopulations of *B.bovis*; it was clear that changes in the ratios of subpopulations occur during attenuation and as a result of *in vitro* cultivation. Amongst the isolates analysed, one designed Lismore (W), which had been cultured *in vitro*, has shown to contain two subpopulations, whereas the other designed Lismore (LP), which originated from the same field infection as the Lismore stock used as the parent line in the present study (P.Timms, personal communication), was shown to contain one genetically homogeneous population. Dalrymple and co-workers also suggested that increase of virulence cannot be associated with selection of a distinct major subpopulation of parasites, since although the Lismore (LP) isolate appeared to be more virulent after being passaged through ticks, no genetic differences were observed. From the genetic characterisation carried out by Dalrymple and co-workers on the Lismore isolate one can infer that the parent line used in the present study contained one single population of *B.bovis* parasites, and this could be one explanation for the similarity of the cloned lines to the parent line when analysed by their growth rates, morphology and GPI isoenzyme patterns. Although clone 4 was used to provide cultured parasite material for evaluation of the effect of different media (section 4.3) and the incorporation of feeder cells on *B.bovis* cultivation (section 4.4), the cloned lines were not further analysed, since it was assumed that all of them comprised the same homogeneous population as the original isolate (parent).

4.5 INITIATION OF *B.BOVIS* CULTURES FROM LOW PARASITAEMIA BLOOD

During this study, one of the calves inoculated with *B.bovis* (Mexico) never showed detectable parasitaemia in smears from blood taken from the jugular vein, although few parasites (not more than 6 parasites/smear) were seen in smears from peripheral blood (ear vein) on two occasions (days 7 and 11 after first infection).

Blood for initiation of cultures of *B.bovis* is usually collected when parasitaemia exceeds 0.1% (Conrad, 1983; L.Bell-Sakyi, personal communication) and the establishment of a culture of Mexico stock from infected blood when the parasitaemia was less than 0.1% had only been successful when a BAE feeder layer was used to initiate cultures (Conrad, 1983). Rodriguez and co-workers have also reported that the standard method for cultivation of *B.bovis* does not serve to initiate cultures from blood with a parasitaemia below 0.1% and have modified some of the culture components, such as gas tension and fluid level in order to allow the establishment of cultures from small numbers of parasites (Rodriguez et al, 1983).

This experiment was designed to examine the possibility of establishing of *B.bovis* cultures from peripheral infected blood with parasitaemia undetectable in Giemsa stained smears, using 4% O_2 gas tension, which is the gas tension used for resuscitation of stabilates.

4.5.1 Experimental design

Blood from calf 396 (see table 3.1) collected on day 12 after infection with *B.bovis* (Mexico) was used to initiate cultures. No parasites were seen in smears made from blood collected from the jugular vein or from peripheral blood (tail tip capillary blood). Approximately 1 ml of heparinized blood was collected by ear vein puncture and was spun at 1,000 x g for 10 minutes at 15° C. Plasma, buffy coat and upper 10% of RBC were discarded. The pellet was resuspended in 1 ml of complete M199-H (see table 3.2) and the suspension was divided between 6 wells of a 96-well plate (200 µl/well) and incubated under 4% O_2 gas. Medium changes (130 µl/well) were carried out daily and smears were made daily initially from 1 well, and from day 5 onwards from all wells.

On day 5 three wells were each subcultured (1 in 2 dilution with fresh RBC suspension) into 4 additional wells in the same plate, which remained under reduced O_2 gas incubation, and three other wells were subcultured into a 24-well plate by adding the entire contents of each well (200 µl) to 1.0 ml of fresh 10% RBC suspension. The 24-well plate was incubated under 5% CO_2 in air. Medium (1.0 ml/well) was changed and smears were made daily from each well.

On day 7 each well in the 96-well plate was subcultured by transferring its entire contents to wells of the 24-well plate and adding 1.0 ml of fresh RBC suspension as described for day 5. On day 8 the contents of 3 wells from the 24-well plate were transferred to 3 flasks (25 cm^2) and 4.0 ml of fresh RBC suspension were added to each flask.

4.5.2 Results

The results from the examination of Giemsa stained smears that all six wells of the 96-well plate contained at least 1 parasite per field 5 days after initiation of cultures, as shown in Table 4.2.

The three flasks originated from wells in the 24-well-plate were cultured as usual with medium changes daily and subcultures (1 in 5) with fresh RBC suspension every 2 days. On day 14 the contents of each flask were cryopreserved (section 3.3.4).

Table 4.2 Assessment of *in vitro* growth of *B.bovis* cultures (Mexico stock) initiated from infected blood with undetectable parasitaemia.

	DAYS				
Well No	**1**	**2**	**3**	**4**	**5**
1	ND	ND	ND	ND	+++
2	ND	ND	ND	ND	+++
3	ND	ND	ND	ND	+++
4	ND	ND	ND	ND	+++
5	ND	ND	ND	ND	+++
6	+	++	+++	+++	+++

ND Not determined

\+ 3 parasites seen in 100 fields

++ 12 parasites seen in 100 fields

+++ at least 100 parasites seen in 100 fields

4.5.3 Discussion

In the present study it was possible to establish cultures of *B.bovis* from low parasitaemia blood (undetectable by Giemsa stained smears) under an initial incubation atmosphere of 4% O_2 tension and a medium depth of 7 - 8 mm. The incubation under reduced O_2 atmosphere was the critical component for the success in establishment of the cultures, as reported by Rodriguez and co-workers (Rodriguez et al, 1983), who also initiated cultures from carrier cattle when no parasites had been seen in blood smears, using reduced O_2 gas tension, but a fluid level of 4 mm, whereas initiation was not possible when a higher O_2 atmosphere was used.

The fact that the blood had been collected from an ear vein might have facilitated the establishment of the cultures, because parasites had been detected in smears made from peripheral blood on two occasions before the initiation of cultures (days 7 and 11 after infection), whereas no parasites had been seen in smears from jugular blood at any time after infection. The chance of detecting *B.bovis* increases when smears are made from peripheral blood rather than from jugular blood, due to the fact that parasitised RBC tend to accumulate in peripheral veins and capillaries (Mahoney, 1977). This has been applied for the diagnosis of acute *B.bovis* infections. Despite the lack of clinical disease, calf 396 developed high titres of specific antibodies, shown by IFAT and ELISA, which increased after each challenge (as described in Chapters 6 and 8). The fact that calf 396 had not been splenectomised probably contributed to such low parasitaemia.

Furthermore, the lack of patent parasitaemia and clinical signs observed might be related to low virulence of the cultured parasites used for the primary infection and subsequent challenges. *In vitro* cultivation of *B.bovis* in the presence of horse serum has been demonstrated to attenuate the parasite (Yunker et al, 1987), and cloning by *in vitro* cultivation always results in less pathogenic lines (Pipano, personal communication). Thus, as the Mexico stock of *B.bovis* used here had been maintained in culture on several occasions since its original isolation, it is possible that less pathogenic parasite populations were selected.

4.6 *IN VITRO* CULTURE OF STOCKS OF *B.BIGEMINA*

4.6.1 Zaria stock

4.6.1.1 Experimental design

A series of experiments was carried out in which blood samples collected from one calf infected with *B.bigemina* (Zaria) blood stabilate (calf 397, as referred in Table 3.1) were used for initiation of cultures under several combinations of culture medium and serum concentrations at two different gas tensions.

The culture media (GIBCO) tested in this study included:

- M199 with Hanks salts and 25 mM HEPES (M199-H) cat No 04102350H

- M199 with Earle's salts and 25 mM HEPES (M199-E) cat No 041-02340H

- M199 with Hanks salts (M199-IC) cat No 041-01151H

- RPMI 1640 with 25 mM HEPES (RPMI) cat No 041-02400H

Cultures were initiated on three consecutive days using jugular blood collected from day 5 to day 7 after infection, when parasitaemias estimated in Giemsa stained smears were less than 0.1%.

For the first attempt to establish cultures (on day 5 after infection), defibrinated and heparinized blood were collected and prior to initiation of cultures the blood was pre-treated with PIGA buffer (Appendix 1) (Montenegro-James, personal communication) by adding one volume of buffer to 10 volumes of blood and incubating the suspension at 37^{o} C for 1 hour. The blood was then spun at 1,000 x g for 10 minutes at 15^{o}C; these centrifugation conditions were used throughout the study, unless otherwise specified. The serum/plasma, buffy coat and upper 20% of RBC were discarded. The erythrocytes were washed twice with M199-H by centrifugation, under the same conditions as above, and RBC suspensions, with PCV of both 5 and 10%, were prepared in either M199-H supplemented with 20 or 40% NBS or in RMPI also supplemented with 20 or 40% NBS. This resulted in 8 different culture media with defibrinated blood and 8 with heparinized blood (4 with PCV of 5% and 4 with PCV of 10% for each type of blood). Each RBC suspension was used to initiate cultures in 24-well plates (1.2 ml/well) incubated under 4% O_2 gas tension. The overlying medium in each well was changed daily by removal and replacement of 1 ml with fresh medium and Giemsa stained thin smears (as described in section 3.3.3) were made daily. Fifty microscope fields from each smear were examined at 500 x magnification for assessment of parasite growth.

For the second initiation of cultures, defibrinated blood was collected (on day 6 after infection) and centrifuged. The serum, buffy coat and upper 20% of RBC were discarded.

The packed RBC were washed twice by centrifugation with PIGA buffer and cultures were set up in 24-well plates (1 ml/well) as a 10% RBC suspension in either M199-H or RPMI 1640 supplemented with the following proportions of NBS:

Culture medium	NBS (%)	PIGA buffer (%)
M199-H	----	10
M199-H	20	20
M199-H	10	----
M199-H	5	----
RPMI	----	10
RPMI	20	20
RPMI	10	----
RPMI	5	----

The decision to use defibrinated blood and culture media with less NBS, was based on the results obtained from the first attempt (as described in 4.2.3). Incubation conditions and assessment of parasite growth were identical to those previously described.

For the third and last attempt to establish Zaria stock of *B.bigemina* in culture, defibrinated blood was collected (on day 7 after infection) and centrifuged. The serum (designated autologous serum) was kept while the buffy coat and upper 20% of RBC were discarded. Half the packed RBC was used to prepare a 10% RBC suspension in autologous serum and the other half was incubated with an equal volume of PIGA buffer at 37°C for 1 hour. The resulting suspension was then centrifuged at 1,000 x g for 10 minutes. The supernatant was removed and the packed RBC (treated with PIGA) were used to prepare a 10% RBC suspension in autologous serum. Each of the RBC suspensions (with and without PIGA treatment) was then diluted with three different media: M199-E, M199-E to which 1.4 g/l of $NaHCO_3$ were added (M199-ES) and RPMI as followed:

Culture medium	10% RBC suspension	additional autologous serum	final concentration of serum (%)
M199-ES (0.90 ml)	0.40 ml	----	28
M199-E (0.45 ml)	0.40 ml	0.45 ml	62
M199-E (0.45 ml) + RPMI (0.45 ml)	0.40 ml	----	28
RPMI (0.45 ml)	0.40 ml	0.45 ml	62

Each aliquot of the final suspension (1.3 ml with PCV of approximately 3%) was used to initiate a culture in a 24-well plate. The plate was incubated under 5% CO_2 in air. After 24 hours of incubation, the overlying medium (~ 1 ml) of each well was removed and the same volume of each corresponding fresh medium replaced. Giemsa stained thin smears were prepared for parasitaemia estimation. These procedures were done over a three day period. Fifty microscope fields of each smear were examined at 500 x magnification.

4.6.1.2 Results

From the examination of Giemsa stained smears taken 24 hours after the first initiation of cultures, it appeared that cultures initiated from defibrinated blood in M199-H supplemented with 20% NBS resulted in survival of higher number of parasites, as shown in Table 4.3. No parasites were seen in cultures with 10% PCV in M199-H supplemented with 40% NBS, whether initiated from defibrinated or heparinized blood; a few parasites survived under these conditions with a 5% PCV. However examination of smears after a 48 hour incubation period, showed that none of the culture media supported parasite growth, regardless of whether heparinized or defibrinated blood with a PCV of 5 or 10% was used to initiate cultures.

In the second attempt to initiate cultures, in which several concentrations of NBS were tested in different combinations with M199-H, RPMI and PIGA buffer, none of the conditions supported parasite survival after 24 hours.

Results from the third attempt to establish cultures showed that all the culture conditions tested supported parasite survival for 24 hours. However a much lower number of parasites were seen in cultures initiated with RBC which had been pre-treated with PIGA, suggesting that the PIGA contributed to parasite killing. Cultures with untreated RBC in M199-ES (M199 with Earle's salts supplemented with 1.4 g/l of $NaHCO_3$) containing approximately 28% of autologous serum, contained more parasites than the other media (Table 4.4). Under these conditions some parasites survived at least for 48 hours. However examination of cultures at 72 hours after initiation showed that none of conditions supported parasite survival.

Table 4.3 Assessment of *in vitro* cultures of *B.bigemina* (Zaria stock) with a PCV of 10% or 5%, under different culture media.

		PCV			
Type of blood	**Culture medium**	**10%**		**5%**	
		24 hours	**48 hours**	**24 hours**	**48 hours**
defibrinated	M 199-H, 20% NBS	++	-	+	-
	M 199-H, 40% NBS	-	-	+	-
	RPMI, 20% NBS	+	-	+	-
	RPMI, 40% NBS	+	-	+	-
heparinized	M 199-H, 20% NBS	+	-	+	-
	M 199-H, 40% NBS	-	-	+	-
	RPMI, 20% NBS	-	-	-	-
	RPMI, 40% NBS	+	-	-	-

Key: + at least 1 parasite seen per smear
++ at least 10 parasites seen per smear
- no parasites seen

Table 4.4 Assessment of *in vitro* cultures of *B.bigemina* (Zaria stock) under different culture media.

RBC	Culture medium	24 hours	48 hours	72 hours
Non-treated	M199-ES, 28% serum	+++	+	-
	M199-E, 62% serum	++	-	-
	M199-E + RPMI-1640, 28% serum	+	-	-
	RPMI, 62% serum	+	-	-
Treated with PIGA	M199-ES, 28% serum	+/-	-	-
	M199-E, 62% serum	+/-	-	-
	M199-E + RPMI-1640, 28% serum	+/-	-	-
	RPMI, 62% serum	+/-	-	-

Key: +++ more than 20 parasites seen
++ 10 to 20 parasites seen
+ 5 to 10 parasites seen
+/- less than 5 parasites seen
- no parasites seen

4.6.2 Muguga stock

4.6.2.1 Experimental design

Blood used for initiation of cultures of *B.bigemina* (Muguga stock) was collected from one calf (calf 634, as referred in Table 3.1) which had been infested with larvae of *Boophilus decoloratus*, as described in section 3.2.2. The larvae were provided by the National Veterinary Research Centre, Kenya, and derived from females which had engorged on an intact calf infected with *B.bigemina* (Muguga) 18 days after ticks were applied to the calf. The ticks used had been shown to be free from all tick-borne diseases by feeding on susceptible calves for two generations. Females were allowed to lay eggs at 28°C and once the larvae had hatched they were kept at 18°C until transport to CTVM. Defibrinated jugular blood was collected from calf 364 when the parasitaemia detected in Giemsa stained smears was less than 0.1% (on days 19 and 20 after larval infestation). The blood was centrifuged and the serum was kept (autologous serum). The buffy coat and upper layer of RBC were discarded as described in previous experiments.

The pelleted RBC were used to prepare 5% RBC suspensions in either autologous serum or in M199-ES and RPMI, both supplemented with 30% autologous serum and cultures were set up in 24 well plates (1.3 ml/well). The plates were incubated under an atmosphere of 5% CO_2 in air. The overlying medium of each well was changed and thin Giemsa stained smears prepared and examined daily over a period of three days.

4.6.2.2 Results

None of the culture conditions tested supported parasite survival, since no parasites were seen at 24 or 48 hours after initiation of cultures.

4.6.3 Mexico stock

The third isolate of *B.bigemina* (Mexico), originally established in culture in 1985 (Vega et al, 1985a), was obtained in 1992 as a growing culture suspension from Imperial College (London) and cultured using the same conditions used at Imperial College, i.e. as a 10% RBC suspension in M199-IC, to which 15 mM HEPES were added, supplemented with 40% NBS, under an atmosphere of 5% CO_2 in air; the overlaying medium was changed daily and subcultures were carried out every 2-3 days. An experiment was carried out at Imperial College, by which several combinations of RBC suspensions and serum samples from three cows maintained at CTVM (110, 16 and Split Ear) were tested in order to identify the most

suitable donor(s) of RBC and serum for maintenance of the cultures at CTVM. Results from this experiment showed that all combinations of RBC and sera supported *in vitro* growth of *B.bigemina*. However, the combination of RBC and serum taken from the two-year-old purebred Jersey (Split Ear) resulted in slightly higher parasitaemias of cultures (A.Gunn, personal communication). Thus, this animal was used for provision of both RBC and sera for maintenance of *B.bigemina* (Mexico) cultures at CTVM.

Typical parasitaemias observed in Giemsa stained smears from growing cultures were 2-3% and 4-7% at respectively 24 hours and 48 hours after subculture. Subcultures were carried out at a dilution of 1 in 5, resulting in reduction of parasitaemia to approximately 1%. Two types of culture vessels were used: 25 cm^2 flasks containing 5 ml of culture suspension, which were incubated vertically, and 75 cm^2 flasks containing 40 ml of culture suspension, which were incubated horizontally.

4.6.4 Discussion

The Mexican isolate of *B.bigemina* obtained from Imperial College was successfully maintained at CTVM as a growing culture, achieving parasitaemias of 4-7%. Cultures of *B.bigemina* were bulked up on several occasions for provision of *i*) culture supernatant, which was analysed by high performance liquid chromatography (HPLC) (described in Chapter 6); *ii*) iRBC, which were concentrated by density gradient centrifugation (described in Chapter 5) and used for preparation of samples for electrophoresis; *iii*) infected cells for metabolic labelling experiments; and *iv*) culture suspension with which conditions for induction of free merozoites were evaluated.

Although many attempts were made to establish cultures of the two African isolates of *B.bigemina* using several combinations of RBC and serum donors, culture media and gas tensions, the conditions used did not allow the establishment of a *B.bigemina* culture system for these two isolates. The source of the NBS did not appear to be a detrimental factor in the culture system, since sera from the same two cows that did not support survival of Zaria and Muguga parasites proved to be suitable for cultivation of the Mexican stock of *B.bigemina*.

After so many unsuccessful attempts, it seems that the major limiting factor was related to the isolates themselves, since none of the conditions tested could support survival of parasites for more than 48 hours and in no case was there any sign of parasite multiplication.

The African isolates used in this study are naturally transmitted to cattle by *Boophilus decoloratus* ticks, while the isolates which have been established *in vitro* so far are naturally transmitted by *Boophilus microplus* (Vega et al, 1985a; Montenegro-James et al, 1987; Jorgensen et al, 1992) or by *B.annulatus* (Fish et al, 1992). This fact might suggest that the Muguga and Zaria isolates are different metabolically from those already cultured and may

have different requirements for an *in vitro* cultivation system. Furthermore, the rate of success in establishing isolates of *B.bigemina* from infected blood as continuous *in vitro* cultures appears to be very low; in Israel, for instance, it has been reported that an average of 30 attempts are required for each successful *in vitro* establishment (E.Pipano, personal communication).

The successful cultivation of the Mexican isolate of *B.bigemina* under conditions very similar to those that had failed to support *in vitro* survival of the two African isolates, associated with the low rates of establishment of new isolates in culture gives further evidence that different isolates may have different requirements for an *in vitro* cultivation system and points to the need for further investigations on physio/metabolic aspects of *B.bigemina* parasites, which could optimise the culture system in order to make it suitable for the establishment of more isolates from the field.

4.7 CONCLUSIONS

The studies on the effect of calf sera on *B.bovis* cultivation showed that sera from calves between 4 and 7.5 months of age, with only one exception, were able to support parasite growth *in vitro* in established cultures over a period of 6 days. The serum from a 6-month-old calf which did not support *B.bovis* growth in one experiment proved to support parasite growth when the same calf was 7.5 months old. The presence of anti-*Babesia* antibodies in the calf sera did not affect *B.bovis* growth *in vitro*. These results suggest that sera from immune calves with antibodies detectable by IFAT might be of use for cultivation of *B.bovis* parasites in endemic areas, where the identification of a suitable seronegative adult donor may constitute the major limitation for the implementation of a culture system.

The attempts to increase parasitaemias of *B.bovis* cultures, by the incorporation of cell feeder layers, showed that BAE, contrary to expectation, did not affect the growth of established cultures. Mouse peritoneal wash cells (MPWC) had no effect on resuscitation of culture stabilates. However, they promoted increase in parasitaemias of established cultures of all three stocks of *B.bovis* when used at concentrations of 10^4 and 10^3 cells/ml. Moreover, the incorporation of MPWC into cultures of the Lismore and Mexico stocks resulted in fewer pycnotic parasites, which are an indication of unhealthy cultures. Nevertheless, the increase in parasitaemias obtained with the incorporation of MPWC in established cultures was much less proportionately than that reported for *Plasmodium* spp (Trenholme & Phillips, 1989) and their use as a promoter of *B.bovis* growth *in vitro* was not considered worthwhile for the purposes of this study. On the other hand, in the present study the MPWC did appear to have a beneficial effect on *B.bovis in vitro*, suggesting that the incorporation of MPWC, as reported for *Plasmodium* spp, might facilitate the establishment of field isolates of *B.bovis* which may contain populations of parasites that are more difficult to grow *in vitro*.

The cloning by limiting dilution of the Lismore stock of *B.bovis* resulted in six cloned lines; however the evaluation of their growth rates, morphology and presence of isoenzymes did not distinguish any of them from the parent line.

The procedure by which one of the cloned lines was recloned was shown to be more accurate than that initially used for cloning, hence the number of positive wells obtained was closer to that expected. However, none of the recloned lines were able to multiply continuously beyond 24 days in culture. This finding indicates that in this case the culture conditions used for initiation of cultures from a single parasite were not appropriate for support of continuous parasite multiplication, and points to the importance of changing the culture conditions as soon as the parasites have begun to multiply appreciably.

Although one cloned line was used as the source of parasites in some experiments, it was assumed on all the evidence available that the cloned lines were not different from each other and therefore the clones were not included in the comparative immunochemical analysis. Furthermore, a study by Dalrymple and colleagues found, by genetic analysis, that the original Lismore isolate contained only a single parasite population (Dalrymple et al, 1992).

A culture of *B.bovis* (Mexico) was successfully established *in vitro* from blood collected from an ear vein of an infected calf, when no parasites were detected in Giemsa stained blood smears. Though no attempt was made to set up cultures from jugular blood at the same time, this might suggest that the use of peripheral blood for initiation of *B.bovis* cultures might facilitate the establishment of field isolates *in vitro*; further investigations with comparison between the success rates for establishment of cultures from jugular and peripheral ear vein blood would be needed to confirm this hypothesis.

The Mexico stock of *B.bigemina* (Vega et al, 1985a), obtained as a growing culture suspension from Imperial College, was successfully maintained continuously *in vitro* at CTVM, with parasitaemias between 4 and 7%. The ability to grow this stock of *B.bigemina* continuously *in vitro* represented an important achievement in the provision of a reliable source of parasites for use during the immunochemical analysis, which was the basis for the identification of species-specific parasite components with potential use as immunodiagnostic targets (as described in Chapters 6 and 7). Although the Mexico stock of *B.bigemina* was obtained late in the present study, it was possible to use it in a series of experiments in which different attempts were made to concentrate parasites or iRBC in order to obtain parasite-rich preparations for use in immunochemical analyses. These attempts are described in Chapter 5.

All the attempts made in the present study to establish the two African isolates of *B.bigemina* (Zaria and Muguga) as continuous cultured lines were unsuccessful, with suggestions of remarkable differences between the Mexican and the African isolates. This was based on the failure of Zaria and Muguga parasites to survive beyond 48 hours under culture conditions very similar to those used for cultivation of the Mexican stock. The lack of success in establishing the African isolates indicates that the culture system developed for *B.bigemina* (Vega et al, 1985) may not be suitable for the establishment of different isolates and points to a need for further studies on the *in vitro* requirements of *B.bigemina* parasites.

CHAPTER FIVE

CONCENTRATION OF *BABESIA* PARASITES

5.1 INTRODUCTION

The *in vitro* culture systems developed for *B.bovis* (Levy & Ristic, 1980) and for *B.bigemina* (Vega et al, 1985a) still provide relatively low percentages of iRBC making necessary the development of methods of parasite concentration in order to obtain parasite-rich material with less host contamination for use in immunochemical studies. These methods have focused on the concentration of either free parasites, or iRBC from which the parasite can be isolated by a lysis process.

Concentration of *B.bovis* iRBC has been achieved by differential lysis of non-infected RBC with hypotonic solutions (Mahoney, 1967a; Kahl et al, 1982a). However the same method has not been successfully applied to *B.bigemina* (Mahoney, 1967b; Wright, 1973b). A procedure for differential lysis of uninfected RBC using KCl, followed by the lysis of concentrated iRBC with NH_4Cl, has been routinely used at CTVM for preparation of crude ELISA antigen of *B.bovis* and samples for electrophoresis analysis (see section 3.6.1.1).The efficacy of these methods has been satisfactory, allowing concentration of parasitized cells. However all the chemical lysis procedures will affect original characteristics of the *Babesia* parasites, both structural and biochemical. Therefore, in cases where the preservation of the original structure of the parasites is desired, such as in studies of merozoite surface components, alternative methods based on accumulation of free merozoites in the culture supernatant have been proposed (Levy & Ristic, 1980; Winger et al, 1987). Such methods, which are based on deprivation of cultures of CO_2, had never been tested at CTVM, and in the present study some modifications to the method reported by Winger and co-workers for *B.divergens* (Winger et al, 1987) were evaluated in an attempt to use the induction of free merozoites as an alternative method of purification of both *B.bovis* and *B.bigemina* parasites.

Alternative methods for concentration of *B.bigemina* infected erythrocytes include a procedure of filtration of infected blood through a dextran sulphate-agarose affinity column, by which eluted suspensions contained up to 95% iRBC (Goodger et al, 1989), and the exposure of iRBC to glycerol-enhanced osmotic shock to free the parasites, which were then concentrated by density gradient centrifugation (Figueroa et al, 1990a).

Density gradient centrifugation has been used as a method for separation and purification of cells, viruses and subcellular particles by exploiting differences in their density or size. The use of colloidal silica suspensions as centrifugation media was first reported by Mateyko and Kopac (1963); however, it was found later that those suspensions were unstable in salt solutions at physiological pH and that they were toxic to cells (Wolff, 1975). The development of Percoll (Pharmacia), which consists of colloidal silica particles of 15-30 nm diameter coated with polyvinylpyrrolidone (PVP), allowed the use of modified colloidal silica solutions to generate density gradients completely non-toxic to cells (Pertoft et al, 1978).

Since then, density gradient centrifugation using Percoll has been used to separate different populations of cells (Segal et al, 1980; Ellis et al, 1984) and also to concentrate parasites such as *Trypanosoma* (Castanys et al, 1984), *Plasmodium* (Kramer et al, 1982; Saul et al, 1982), *Anaplasma* (Davis et al, 1978) and sporozoites of *Theileria* from ticks (Walker & McKellar, 1981; 1983).

Percoll has also been used to concentrate *Babesia* parasites. Vega and co-workers reported that *B.bigemina* iRBC were concentrated at least 20 times by Percoll and Percoll-Renografin density gradients and that erythrocytes containing pairs of parasites were concentrated at a density between 1.076 and 1.089 g/ml, whereas erythrocytes containing a single parasite were concentrated at a density between 1.092 and 1.100 g/ml (Vega et al, 1986b). Later the same method was used to concentrate *B.bigemina* iRBC which were then lysed by glycerol-enhanced osmotic shock making possible the purification of erythrocytic stages of that parasite (Figueroa et al, 1990a). A similar use of Percoll density gradient centrifugation has been reported for concentration of *B.bovis* free merozoites and iRBC (Rodriguez et al, 1986a). In that study, the free merozoites were concentrated at a point corresponding to about 1.087g/ml specific density, whereas most of the iRBC were positioned between 1.121 and 1.123 g/ml. More recently, Bhushan and co-workers reported the use of Percoll gradients to concentrate *B.caballi* iRBC, which were located at the interface between densities of 1.080 and 1.115 g/ml (Bhushan et al, 1991).

There are several procedures by which Percoll can be used to generate density gradients. The two procedures used in the experiments described in this chapter were: *i*) a discontinuous gradient, by which solutions of different densities are prepared and carefully layered one on top of another, usually starting with the heaviest, and the cell preparation is then layered on top of the gradient, followed by centrifugation at usually between 400 and 2,000 x g; and *ii*) a continuous gradient formed *in situ* during the centrifugation, by which a solution of Percoll with a particular density is prepared, mixed with the cell preparation and the mixture is centrifuged in an angle-head rotor at a high speed (> 25,000 x g). The density gradient pattern is dependent on the initial density of the solution, characteristics of the rotor and running conditions. Usually a tube containing density markers is used to monitor the gradient formation.

The experiments described in this chapter were designed to compare the efficiency of the accumulation of free merozoites in culture supernatant with both discontinuous and continuous density gradient centrifugation to concentrate RBC infected with *B.bovis* and *B.bigemina* for provision of parasite-rich preparations for immunochemical studies.

5.2 INDUCTION OF FREE MEROZOITES IN CULTURE SUPERNATANT

5.2.1 *B.bovis*

5.2.1.1 Materials and methods

Five ml of a *B.bovis* (Lismore) culture, as a 10% RBC suspension in complete M199-H (see Table 3.2) with a parasitaemia of 5%, were left in air at room temperature in a glass universal container for 4 hours. The culture was spun at 200 x g for 10 minutes at 4°C. The pellet (P1) was retained and the supernatant (S1) was spun at 400 x g for 10 minutes at 4°C. The upper 3 ml of the second supernatant (S2) and the second pellet (P2) were retained and S2 was spun again at 1,000 x g for 20 minutes at 4°C. The final supernatant (S3) and final pellet (P3) were retained. Cytospin smears (as described in section 3.3.3) were prepared from each of the supernatants and blood smears were prepared from each of the pellets.

The final pellet (P3) was resuspended in 600 µl of a 10% RBC suspension in complete M199-H (see Table 3.2) and divided between 3 wells of a 96-well plate (200 µl/ well) in order to check the infectivity of the free merozoites. The plate was incubated under 5% CO_2 in air and medium was changed daily (130 µl/well). From day 2 smears were made daily from each well.

The same procedure of induction of free merozoites was repeated using 5 ml of a *B.bovis* (Lismore) culture suspension which had been left in air at room temperature overnight (approximately 16 hours).

5.2.1.2 Results

The examination of smears showed that when the culture suspension was deprived of CO_2 overnight more free merozoites were released into the culture supernatant than when a 4 hour period was used. This was inferred from the detection of fewer intact iRBC in the first pellet (P1) from cultures left overnight without CO_2 than from cultures which had been left for 4 hours.

The examination of smears from each of the supernatants and pellets after both CO_2 deprivation times showed the following results:

S1	high concentration of free merozoites + RBC membranes + few intact uninfected RBC
P1	intact RBC (infected + uninfected)
S2	high concentration of free merozoites + RBC membranes
P2	some free merozoites + RBC membranes + few intact RBC
S3	RBC membranes
P3	high concentration of free merozoites + RBC membranes

The final pelleted free merozoites (P3) after both CO_2 deprivations were shown to be infective, with all three wells in each of the 96-well plates showing parasite growth after 2 days of culture.

5.2.2 *B.bigemina*

5.2.2.1 Materials and methods

Two 5 ml aliquots of *B.bigemina* (Mexico) culture suspension, as a 10% RBC suspension in M199-IC (see Table 3.2), with a parasitaemia of 7%, were left in air in plastic bijou containers for 6 hours either at room temperature or at 37°C. Both cultures were then centrifuged at 200 x g for 10 minutes at 4°C. The supernatants (S1b) and the pellets (P1b) were retained and S1b supernatants were spun at 400 x g for 5 minutes at 4°C. The upper 3 ml of each supernatant (S2b) and the pellets (P2b) were retained and S2b supernatants were spun again at 1,500 x g for 20 minutes at 4°C. The final supernatants (S3b) and final pellets (P3b) were retained. Cytospin smears were prepared from each of the supernatants and blood smears were prepared from each of the pellets.

5.2.2.2 Results

The results obtained showed that when the culture suspension was deprived of CO_2 at room temperature more free merozoites were released into the culture supernatant than when it was deprived of CO_2 at 37°C. This was inferred from detection of fewer intact iRBC in the first pellet (P1b) from cultures left at room temperature than from the culture which had been deprived from CO_2 at 37°C. However the accumulation of free merozoites of *B.bigemina* in the culture supernatant after the cultures had been deprived of CO_2 for a period of 6 hours was not very efficient, since a large proportion of parasites remained inside erythrocytes after CO_2 deprivation.

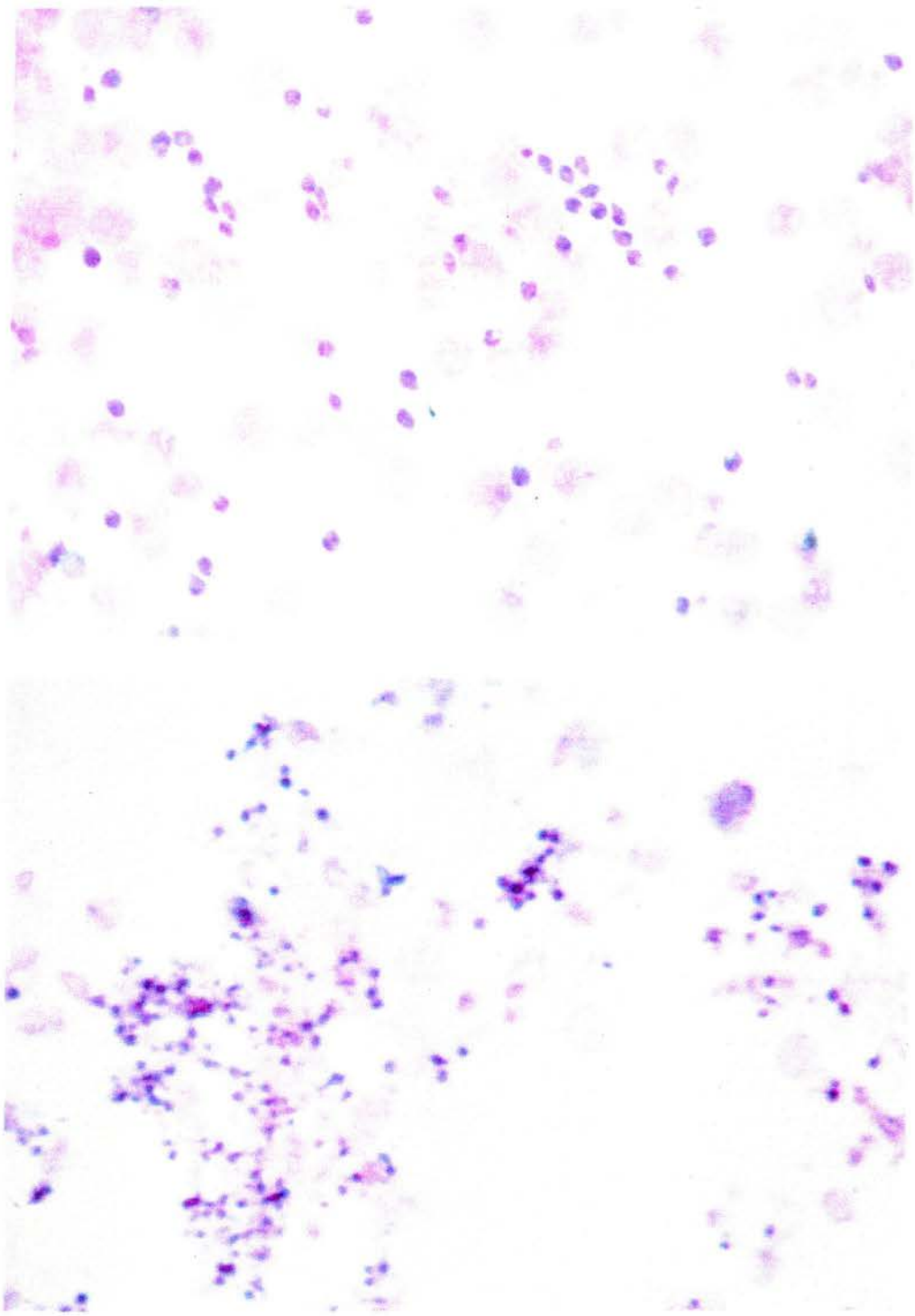
The examination of smears from each of the supernatants and pellets after both CO_2 deprivation conditions showed results very similar to that obtained with *B.bovis* and are presented below:

S1b	high concentration of free merozoites + RBC membranes + few intact uninfected RBC
P1b	intact RBC (infected + uninfected)
S2b	high concentration of free merozoites + RBC membranes
P2b	RBC membranes + few intact RBC + few free merozoites
S3b	RBC membranes
P3b	high concentration of free merozoites + RBC membranes + few intact RBC

Cytospin smears of culture supernatants (S1 and S1b) of *B.bovis* and *B.bigemina* after CO_2 deprivation are shown in Fig 5.1.

Fig 5.1 Cytospin smears of culture supernatant after CO_2 deprivation. A is from a *B.bovis* culture and B is from a *B.bigemina* culture.

Fig 5.1



A

B

5.3 USE OF DENSITY GRADIENTS TO CONCENTRATE INFECTED RED BLOOD CELLS

5.3.1 Continuous gradients

5.3.1.1 Materials and methods

a. Experiment 5.1 (*B.bigemina* infected blood)

A 90% (v/v) isosmotic Percoll solution (P-iso 90) was prepared by mixing 90 volumes of stock Percoll (Pharmacia) with 10 volumes of sterile 1.5M NaCl to obtain a density of 1.123 g/ml (manufacturer's recommendation). The P-iso 90 solution was adjusted to a density of 1.095 g/ml by mixing 10 volumes of P-iso 90 with 3.146 volumes of 0.15M NaCl. The resulting solution had a concentration of 68.46% (v/v) Percoll (P-iso 68.46). Approximately 9.5 ml of P-iso 68.46 solution were used to fill each of 4 polycarbonate tubes (15 x 75 mm).

Jugular blood from a splenectomised calf 583 taken 5 days after infection (see Table 3.1), showing a parasitaemia of 0.6% *B.bigemina* (Zaria)-infected RBC, was defibrinated and centrifuged at 1,000 x g for 10 minutes at 4° C. Serum, buffy coat and the upper 10% of packed RBC were discarded. The remaining RBC were washed with PBS by centrifugation as described before. Approximately 1.0 ml of the packed washed RBC was added to each of 3 tubes containing P-iso 68.46. One ml of a mixture of density marker beads (Pharmacia, 1.098, 1.087, 1.076 and 1.064 g/ml) was added to the 4th tube. The tubes were then sealed. One each of the three RBC tubes was centrifuged, with the marker bead tube, at 30,000 x g at 4° C for 7, 13 or 26 minutes respectively, in a Beckman L-8-60M ultracentrifuge, using a fixed angle rotor (type 65), without braking. Representative layers were collected using a syringe and needle and the cells were washed by centrifugation (2,000 x g for 15 minutes at 4°C) with 20 ml of PBS to remove the Percoll. Cytospin smears (as described in section 3.3.3) were made and examined.

The same procedure was repeated with blood taken 7 days after infection, when the parasitaemia was 0.4%. Four tubes containing P-iso 68.46 (3 for *B.bigemina* packed RBC and 1 for marker beads) were set up exactly as described before and were centrifuged at 8, 12 or 16 minutes under the same conditions as described before. Collection and examination of representative layers were carried out as described above.

b. Experiment 5.2 (*B.bigemina* culture suspension)

For this experiment P-iso 68.46 was prepared as described in experiment 5.1 and was used to fill 12 tubes (polyallomer tubes, 5/8" x 3", 11 ml capacity). One hundred and twenty ml of *B.bigemina* (Mexico) culture suspension, with a parasitaemia of 5% and harvested at 48

hours after subculture, were centrifuged at 1,000 x g for 10 minutes at 4°C. The culture supernatant was discarded and 1 ml of the packed RBC was added to each of 11 tubes. One ml of a mixture of marker beads, which included one extra density marker of 1.051 g/ml to the ones already used in experiment 5.1, was added to the 12th tube. The RBC suspension used for the *B.bigemina* cultures had previously been cleared of leucocytes aseptically by passage through a sterile glass column containing cellulose powder (CF-11) (as described for preparation of *B.bovis* samples for electrophoresis in section 3.6.1.1). The 12 tubes were centrifuged at 30,000 x g for 13 minutes at 4°C in a Beckman ultracentrifuge, using a fixed angle rotor (type 70 Ti), without braking. The resulting 8 bands (see Fig 5.4) from each of the 11 tubes were collected, pooled to give 8 samples and the cells washed by centrifugation with PBS as described before. Cytospin smears were made from each of the 8 samples.

5.3.1.2 Results

The formation of the density gradients after 7, 8, 12, 13, 16 and 26 minutes of centrifugation, monitored by the coloured marker beads, is represented in Fig 5.2.

The appearance of the density gradients obtained after centrifugation with *B.bigemina* infected blood (experiment 5.1) is represented in Fig 5.3. The results from cytospin smears showed that under the conditions used the most suitable centrifugation time for separating infected from uninfected RBC was 13 minutes, by which time most of the *B.bigemina* iRBC were concentrated at a specific density between 1.064 and 1.076 g/ml. Cytospin smears made from that specific density layer (shown in Fig 5.4) had a parasitaemia of approximately 90% and a few free merozoites were present. After a centrifugation period of 16 minutes, fewer iRBC and more free merozoites were seen in a pale layer which had a density close to 1.076 g/ml. The majority of the uninfected RBC were concentrated at densities higher than 1.087 g/ml in all centrifugation times.

The density gradients obtained from centrifugation of *B.bigemina* culture suspension (experiment 5.2) are represented in Fig 5.5. The cytospin smears showed that most of the iRBC were located in 4 layers which had densities corresponding to the layer between 1.087 and 1.098 g/ml in the marker bead tube. However, concentration of infected RBC was also detected in other layers located at densities between 1.076 and 1.087 g/ml. Nearly all uninfected RBC were concentrated at a density higher than 1.098g/ml.

Fig 5.2 Development of gradients of a 68.46% Percoll solution during centrifugation at 30,000 x g for 7, 8, 12, 13, 16 and 26 minutes. Gradient densities were monitored by coloured marker beads. (Experiment 5.1).

Fig 5.2

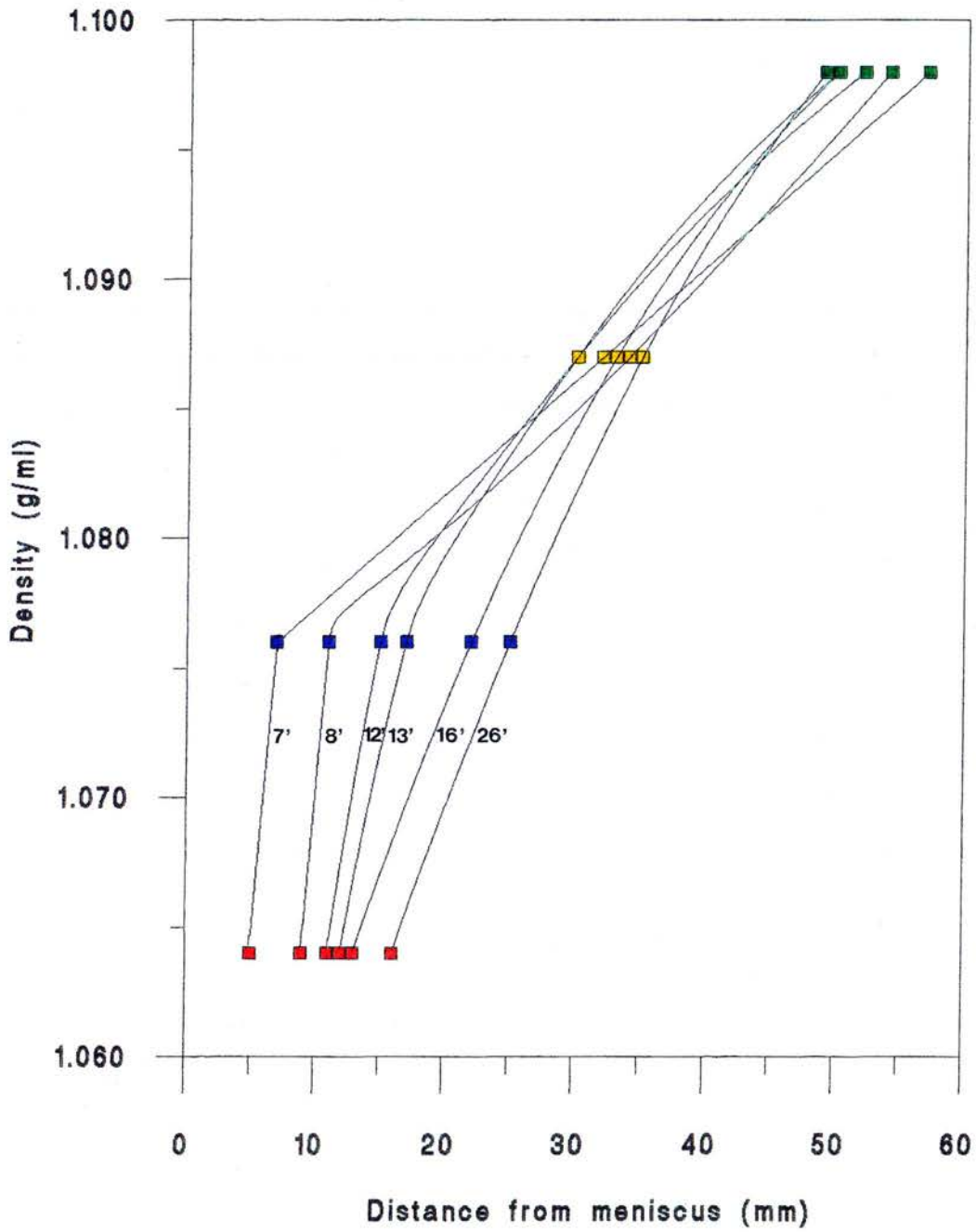
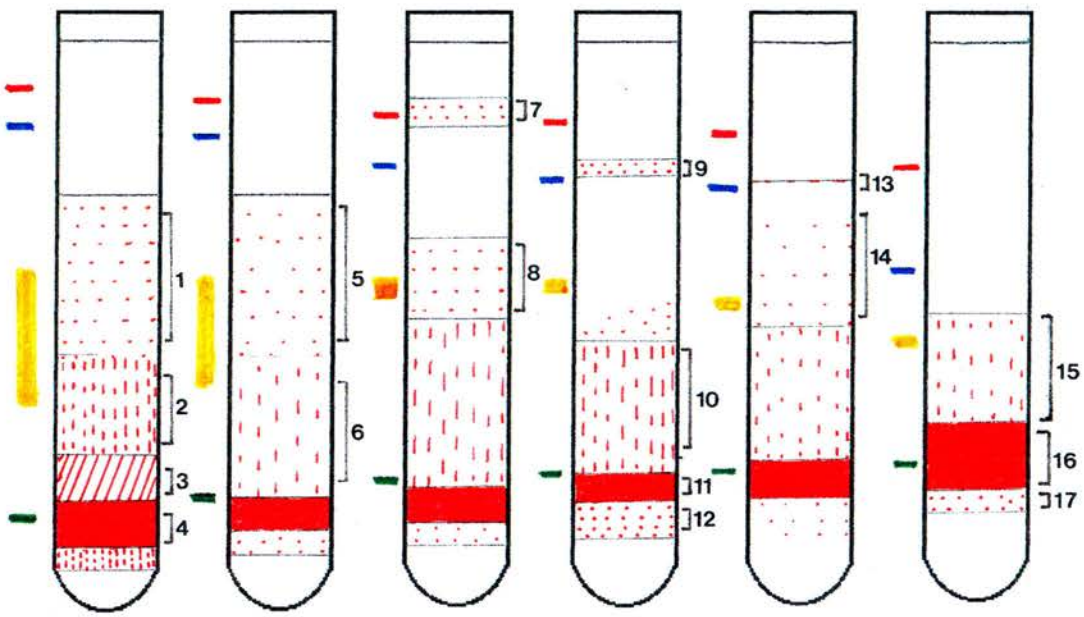

1.100
1.090
1.080
1.070
1.060
Density (g/ml)
7'
8'
12'
13'
16'
26'
0
10
20
30
40
50
60
Distance from meniscus (mm)

Fig 5.3 Diagrammatic representation of continuous density gradients obtained after centrifugation of *B.bigemina* infected blood (starting parasitaemia of 0.6%) at 30,000 x g for 7, 8, 12, 13, 16 and 26 minutes (Experiment 5.1). Densities of marker beads are indicated on the left.

Fig 5.3



7 min	8 min	12 min	13 min	16 min	26 min
1- iRBC (20%) + uRBC + fm	5- iRBC + uRBC	7-iRBC + few uRBC	9- iRBC (90%)	13- fm + few iRBC + uRBC + wc	15-iRBC + uRBC
2- iRBC (1%) + uRBC + RCM + few fm	6- uRBC	8- iRBC + uRBC	10-iRBC + uRBC	14- iRBC + uRBC	16- uRBC
3- uRBC			11- uRBC		17- uRBC
4- uRBC			12- uRBC + few iRBC		

Key:

iRBC - infected red blood cells

uRBC - uninfected red blood cells

RCM - red blood cell membranes

fm - free merozoites

wc - white cells

— 1.064 g/ml

— 1.076 g/ml

— 1.087 g/ml

— 1.098 g/ml

Fig 5.4 Cytospin smear of concentrated *B.bigemina* infected erythrocytes collected from a layer corresponding to a density between 1.064 and 1.076 g/ml (layer 9 in Fig 5.2) from a continuous density gradient after centrifugation at 30,000 x g for 13 minutes (Experiment 5.1).

Fig 5.4

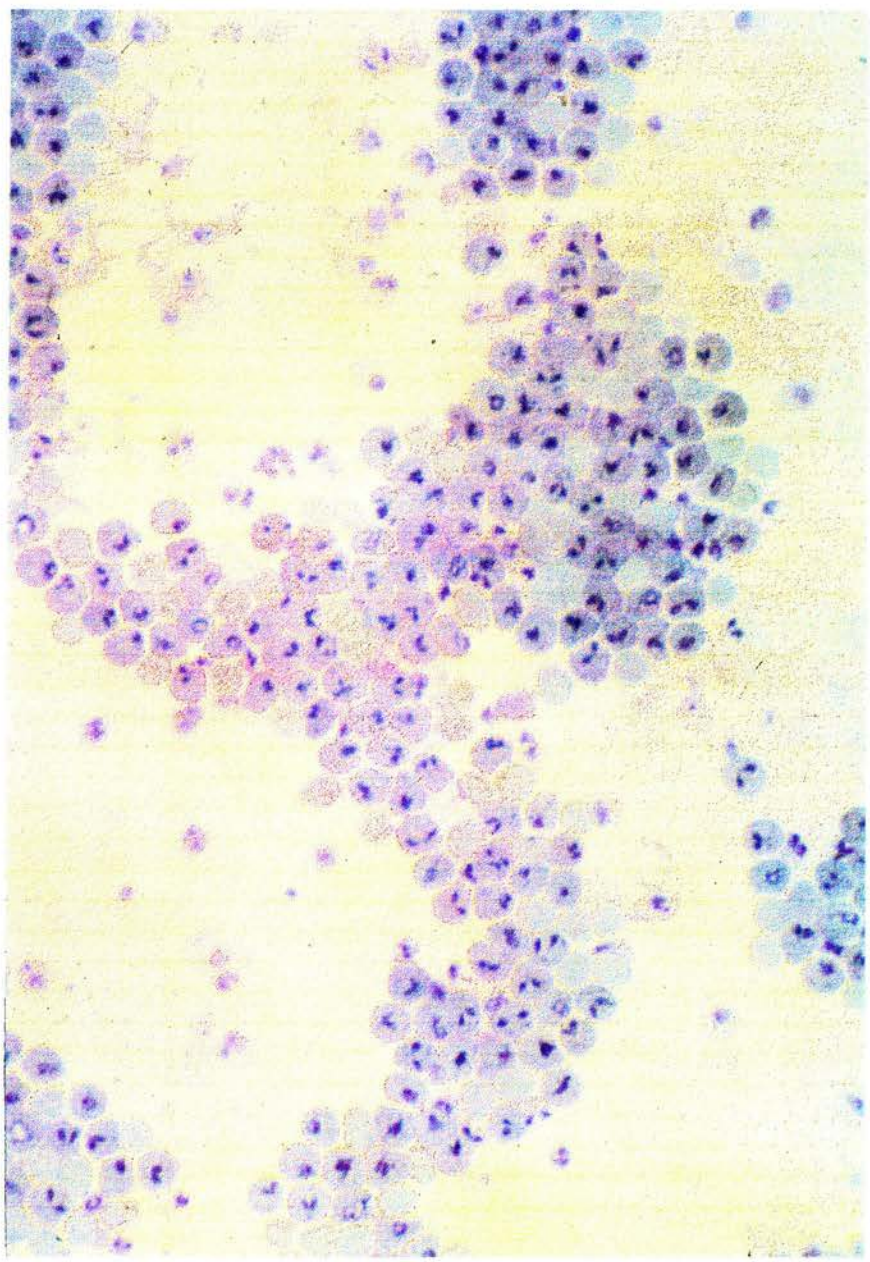
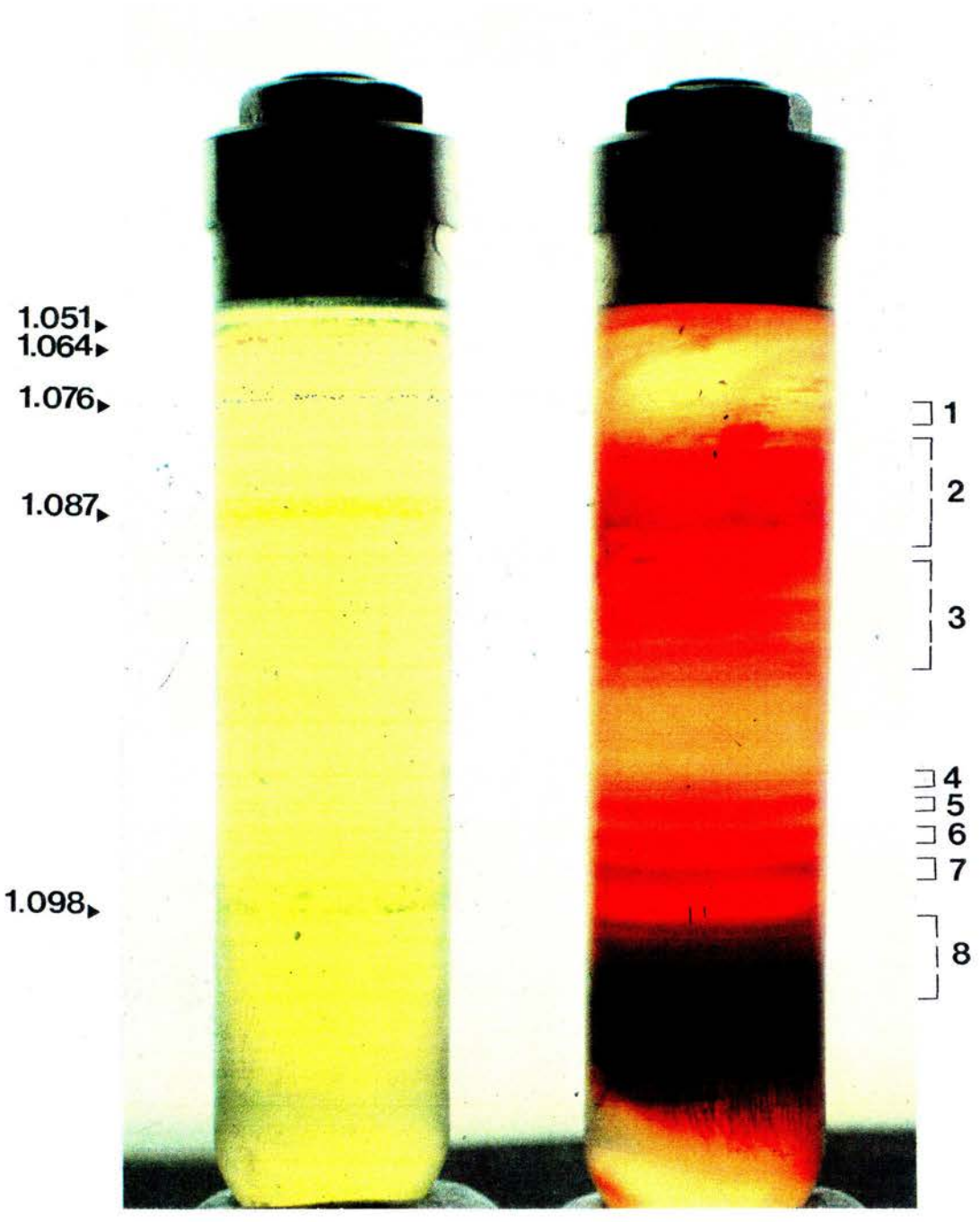


Fig 5.5 Photograph of a tube containing marker beads (A) and a tube containing *B.bigemina* culture material (B) after continuous density gradient centrifugation (Experiment 5.2) .

1- leucocytes
2- leucocytes + few infected RBC
3- few infected RBC
4, 5, 6 and 7 - infected RBC (~90%)
8- uninfected RBC

Fig 5.5



5.3.2 DISCONTINUOUS GRADIENTS

5.3.2.1 Materials and methods

a. Experiment 5.3 (*B.bigemina* infected blood)

Dilutions of Percoll in Hanks balanced salt solution (HBSS) (GIBCO) with bovine plasma albumen (BPA, Sigma) were prepared as follows:

a) 0.5 g of BPA and 0.001 g of sodium bicarbonate were added to 5 ml of 10 x concentrated HBSS. The solution (HBSS/BPA) was sterilised by filtration through a 0.22 μm filter (Millipore)

b) a stock isotonic Percoll solution (SIP) was prepared by mixing 20 ml of sterile Percoll with 2 ml of the HBSS/BPA and the pH was adjusted to 7.2 with sterile 1M HCl

c) 2 ml of the HBSS/BPA solution were added to 18 ml sterile distilled water to give a 1 x HBSS solution with 1% BPA (Hanks/BPA)

d) based on results obtained in experiment 5.1, a range of step dilutions were prepared by mixing SIP and Hanks/BPA in order to give the required densities as followed:

Density (g/ml)	SIP (ml)*	Hanks/BPA (ml)**
1.050	3.6	6.4
1.070	5.4	4.6
1.095	7.6	2.4
1.121	10.0	----

* density of 1.121 g/ml

** density of 1.010 g/ml

Five ml of each step density were layered in each of 2 universal tubes at an angle of 45^{o} in a modified rack, in order of decreasing density starting with the most dense step (1.121 g/ml). Defibrinated blood taken from calf 583 on day 5 after infection, showing a parasitaemia of 0.6%, was centrifuged and prepared as described in experiment 5.1. Two and a half ml of packed RBC were resuspended in 2.5 ml of Hanks/BPA and were layered onto the top of each tube containing the step gradients. The tubes were centrifuged at 4^{o} C, one at 750 x g for 15 minutes and the other at 1,500 x g for 30 minutes in a swing-out rotor. The bands obtained were collected using a Pasteur pipette and the cells were washed by centrifugation with PBS as described before. Cytospin smears were made from each band.

b. Experiment 5.4 (*B.bovis* culture suspension)

A previous report on concentration of *B.bovis* iRBC and free merozoites by the use of Percoll gradient centrifugations showed that they concentrated at densities ranging between 1.087 and 1.123 g/ml (Rodriguez et al, 1986a). Thus, the following step density solutions (as described in experiment 5.3) were prepared for the present experiment:

Density (g/ml)	SIP (ml)	Hanks/BPA (ml)
1.060	4.5	5.5
1.105	8.5	1.5
1.121	10.0	----

Two ml of each density were layered in a universal tube, starting with the most dense step, as described in experiment 5.3. Ten ml of a *B.bovis* culture suspension, with a parasitaemia of 5.5%, were centrifuged at 1,000 x g for 10 minutes at 4°C. The supernatant was discarded and the packed RBC were resuspended in 1.0 ml of Hanks/BPA, and carefully layered onto the top of the step density gradient. The tube was centrifuged at 1,500 x g for 1 hour at 4° C. Cytospin smears were made from each resulting layer.

5.3.2.2 Results

a. Experiment 5.3 (*B.bigemina* infected blood)

Discontinuous gradient centrifugation at 1,500 x g for 30 minutes with *B.bigemina* infected blood resulted in 3 bands, which were identified by their position from the top to the bottom of the tube. The first one was pale and located below the least dense step (1.050 g/ml). The second was a narrow pink band which was followed by a third red condensed band, both located at the interface between the two most dense steps (1.095 and 1.121 g/ml) (Fig 5.6A and 5.6B). The examination of cytospin smears showed that the first band (1) contained a few free merozoites, a few iRBC and a few uninfected RBC. The second band (2) contained uninfected RBC and the third band (3) contained mainly uninfected RBC and very few infected cells.

After centrifugation of *B.bigemina* infected blood at 750 x g for 15 minutes , no interfaces were seen between any of the density steps; however two bands were located within two intermediate density steps (1.095 and 1.070 g/ml) (Fig 5.6C). The first was red and condensed and the second also red but less condensed. Cytospin smears from the first band revealed the presence of uninfected RBC and some free merozoites at the top (4), and

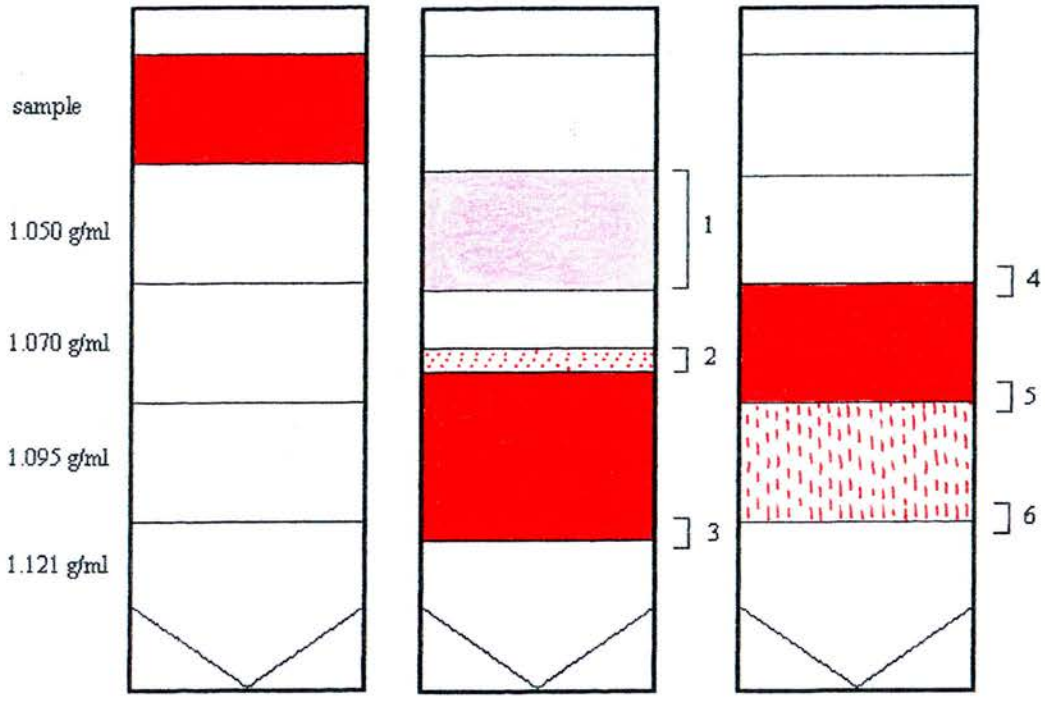
uninfected RBC at the bottom of the band (5). The second band contained uninfected RBC and a few merozoites (6). Separation of *B.bigemina* iRBC after centrifugation at 750 x g for 15 minutes was even poorer than at 1,500 x g for 30 minutes and concentration of *B.bigemina* iRBC or free merozoites was not achieved.

b. Experiment 5.4 (*B.bovis* culture suspension)

The results obtained from *B.bovis* culture suspension are presented in Fig 5.7. The results from cytospin smears showed that a few free merozoites and some uninfected RBC were present in the 1.060 g/ml density step (1). Most of the RBC were located at the interface between the two most dense steps (1.121 and 1.105 g/ml). Two bands were seen at this interface: the first (4) contained mainly iRBC with pycnotic parasites and the second (5) contained iRBC with a parasitaemia of approximately 8% with pairs or single parasites. A few uninfected RBC were also seen in the most dense step (6).

Fig 5.6 Diagrammatic representation of a discontinuous density gradient showing tubes with *B.bigemina* infected blood before (A) and after centrifugation at 1,500 x g for 30 minutes(B) or at 750 x g for 15 minutes (C).

Fig 5.6



1 - few free merozoites + few iRBC + few uninfected RBC

2 - uninfected RBC

3 - uninfected RBC + very few iRBC

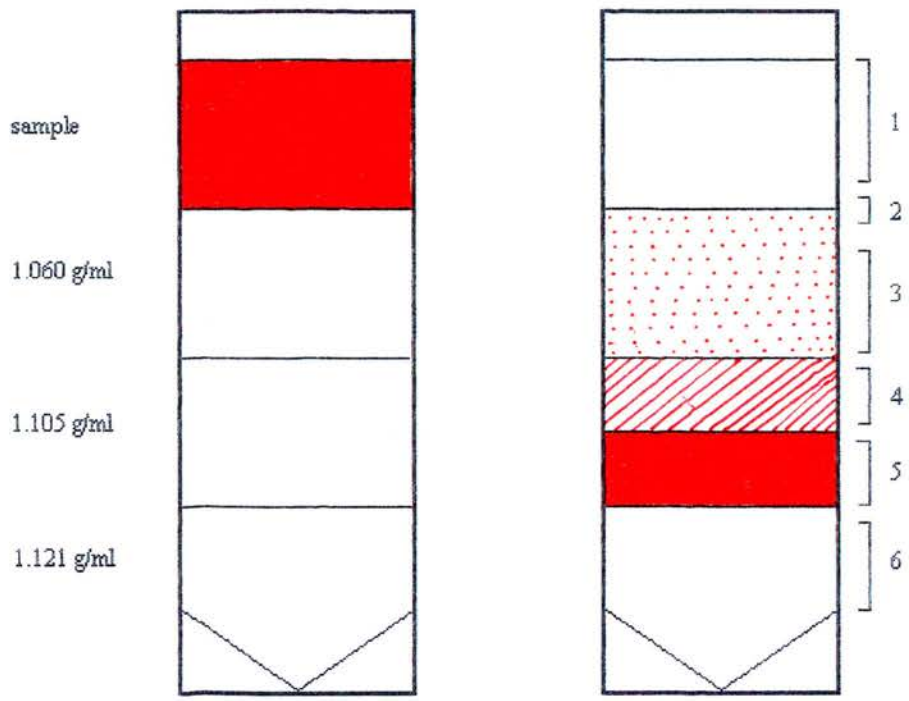
4 - uninfected RBC + free merozoites

5 - uninfected RBC

6 - uninfected RBC + free merozoites

Fig 5.7 Diagrammatic representation of a discontinuous density gradient showing tubes with *B.bovis* cultured material before (A) and after centrifugation at 1,500 x g for 30 minutes (B).

Fig 5.7



(A) **(B)**

1- RBC membranes

2 - free merozoites + uninfected RBC

3 - uninfected RBC

4 - iRBC (~0.5% mainly pycnotic forms)

5 - iRBC (~8%) + uninfected RBC

6 - few uninfected RBC

5.4 DISCUSSION AND CONCLUSIONS

The results of the present study in which merozoites were released suggested that for *B.bovis* a longer period of CO_2 deprivation (overnight) at a lower temperature (room temperature) resulted in release of higher numbers of free merozoites in the culture supernatant. The resulting merozoites successfully infected RBC cultures, indicating the likelihood that the free merozoites survived the separation procedure. Although the possibility that intact iRBC were present in the final merozoite pellet cannot be excluded, they were not seen in Giemsa stained smears.

The first centrifugation of either *B.bovis* or *B.bigemina* culture supernatants at 200 x g for 10 minutes was enough to pellet most of the intact RBC leaving all the free merozoites in the supernatant. The centrifugation of the second *B.bovis* supernatant (400 x g for 10 minutes) resulted in a supernatant with a high concentration of free merozoites and RBC membranes. However some of the free merozoites were pelleted under these centrifugation conditions. For this reason, the length of time used for the second centrifugation of *B.bigemina* culture supernatants was reduced to 5 minutes. Under these conditions, the number of free merozoites pelleted was much lower. The last centrifugation, which was intended to pellet the free merozoites of *B.bovis* (1,000 x g for 20 minutes), had also been modified to 1,500 x g for 20 minutes for *B.bigemina* supernatants and seemed to be adequate to pellet all free merozoites.

The induction of free merozoites of *B.bovis* in culture supernatant proved to be efficient, resulting in a preparation that, although still partially contaminated with RBC membranes, could be useful for immunochemical studies. Besides, the ability to induce free merozoites facilitates studies on morphological, physiological and immunological aspects of this stage of the parasites, which have been reported to contain the major antigenic determinants associated with the induction of protective immunity (James et al, 1981; Ristic & Levy, 1981, Ristic et al, 1981).

Deprivation of CO_2, under the conditions used in the present study, appeared to be less efficient for induction of free merozoites of *B.bigemina* in the culture supernatant, with a large proportion of iRBC remaining intact at the end of the procedure. It was thought that a longer period of CO_2 deprivation was required for induction of more free merozites of *B.bigemina* in the culture supernatant and therefore, on one occasion, 5 ml of *B.bigemina* (Mexico stock) culture suspension was left in air at room temperature overnight. However, after an overnight CO_2 deprivation period, only a small decrease in the number of intact iRBC was detected in smears made from the pelleted cells after centrifugation, and the proportion of parasites that remained inside RBC was higher than that observed with *B.bovis* after overnight CO_2 deprivation. In addition, the free merozoites of *B.bigemina* obtained from all CO_2 deprivation procedures, particularly overnight, appeared fuzzy, with ill-defined

morphology (as shown in Fig 5.1 B), suggesting that either they had suffered damage during the process of release, or that they had subsequently died. In contrast, the free merozoites of *B.bovis* obtained from all CO_2 deprivation procedures appeared to be healthy and completely free from host cell membranes. The observations on *B.bigemina* free merozoites were based only on morphological examination of Giemsa stained smears and no further evaluation concerning their viability or capability to invade other RBC was carried out. Thus, the results obtained in the present study indicate that the induction of free merozoites by CO_2 deprivation may be used as an efficient method for purification and concentration of *B.bovis* parasites from cultures; however it is a less efficient method for concentration of *B.bigemina*, pointing to the need for development of alternative, more efficient methods for the provision of concentrated material from this parasite.

Density gradient centrifugation has been used for many years as a method for separation and purification of cells and particles by which their differences in density and/or size are exploited using a gradient medium. The following characteristics would be desired for an ideal medium (Pharmacia, 1985): it should

- cover a sufficient density range for the particles of interest
- possess physiological ionic strength and pH
- be iso-osmotic throughout the gradient
- be of low viscosity
- be non-toxic
- be unable to penetrate biological membranes
- be supplied sterile and be resterilisable
- be able to form self-generated gradients by centrifugation at moderate g forces
- be compatible with biological materials
- have no adverse effect on assay procedures
- be easy to remove from purified materials
- have no quenching effects on radioactivity

Percoll seems to fulfil all these criteria and it has been used in a number of studies for different purposes (Kramer et al, 1982; Walker & McKellar, 1983; Ellis et al, 1984). Separation of particles or cells by density gradient centrifugation is based on their sedimentation rates, which are proportional to the force applied. The sedimentation rate is directly proportional to the size of the particle and the difference between its density and the density of the surrounding medium. In addition, the concentration of Percoll, the length of time of centrifugation, the rotor geometry and the size of the tubes determine the shape of the gradient density curve, resulting in different arrangements of bands seen in the centrifuge tubes.

The results obtained from the experiments described in this chapter, in which density gradients were used to concentrate iRBC, revealed that concentration of *B.bigemina* iRBC was far more efficient by continuous gradient centrifugation than by using discontinuous pre-formed gradients. By using a 68.46% Percoll solution centrifuged at 30,000 x g for 13 minutes in a fixed angle rotor, a parasitaemia of approximately 90% was achieved from blood with a starting parasitaemia of 0.6%. This result represents a 150 times concentration, which is much higher than the 20 times achieved by Vega and co-workers, who used a continuous Percoll gradient with the same starting density to concentrate *B.bigemina* iRBC (Vega et al, 1986b). In the present study all stages of the parasite were seen at a density between 1.064 and 1.076 g/ml when infected blood with low parasitaemia (0.6%) was used, whereas a wider and higher range of densities (1.076 to 1.098 g/ml) was found to contain concentrated infected erythrocytes when culture suspensions (parasitaemia of 5%) were used. The latter results were closer to that reported by Vega and co-workers, who found that the erythrocytes containing pairs of parasites were located between densities of 1.076 and 1.089 g/ml whereas the ones containing single parasites were located between 1.092 and 1.100 g/ml (Vega et al, 1986b). These authors used for their separation culture suspension with parasitaemias ranging from 2 to 8%, which were comparable to that used in the present study. However, several factors appear to affect the separation procedure; these include the stage of development of cultures at the moment they were harvested - according to Vega and co-workers, cultures taken less than 48 hours or more than 72 hours after subculture yielded fewer infected erythrocytes (Vega et al, 1986b). The lack of separation of paired and single parasite forms into bands of different density in the present study may be due to the fact that here the culture suspension used was harvested at 48 hours after subculture, when predominantly paired parasites were present, while Vega and co-workers used a culture suspension harvested 72 hours after subculture. Furthermore, due to the fact that minor changes in the conditions used resulted in different patterns of separation, one could expect differences even if the same procedure is performed in different laboratories. Interestingly the resulting multiple layers of parasitized RBC between densities of 1.061 and 1.103 g/ml, reported by Vega and co-workers (Vega et al, 1986b), when the time of centrifugation was increased to 30 minutes, were similar to that obtained in the present study using a centrifugation time of 13 minutes (Fig 5.3), by which several layers containing concentrated infected erythrocytes were seen between densities of 1.087 and 1.098 g/ml.

The conditions which had resulted in greatest concentration of *B.bigemina* iRBC in experiment 5.1 were used for concentrating cultured iRBC in experiment 5.2. However considerable differences were found between the band patterns obtained on the two occasions. This may be due to several factors: first of all, although the starting density of the Percoll solution, the speed and the time of centrifugation were the same, a different centrifuge and

rotor were used resulting in differences in the gradient formation, as monitored by coloured marker beads (Fig 5.2 and 5.4); secondly and perhaps the most relevant difference between the two experiments was the origin of the RBC, since in experiment 5.1 calf blood was used whereas in experiment 5.2 adult RBC were used and they might differ in density; thirdly, the fact that the RBC used in experiment 5.2 had been cultured *in vitro* may have resulted in substantial physiological changes which could had affected their density.

The success in separation of *B.bigemina* infected cells from uninfected RBC by density gradients may be related to the preference of that parasite for invading young erythrocytes (Wright & Kerr, 1974). Studies on separation of human RBC have shown that there is an increase in erythrocyte density with age, probably related to a reduction in concentration of several holoenzymes in aged RBC (Spooner et al, 1979). There is a progressive loss of membrane and water during the cell aging process; consequently, at the end of their life span, due to a higher concentration of solutes, RBC are heavier than the youngest cells that enters the circulation as reticulocytes (Piomelli & Seaman, 1993).

The use of Percoll continuous gradient centrifugation has also been reported to purify *B.bigemina* merozoites which had been obtained after chemical lysis of concentrated iRBC by osmotic shock with glycerol (Figueroa et al, 1990a). By that technique only 10% of the parasites were released from the infected erythrocytes after the glycerol treatment, but concentration of free merozoites was obtained in a diffuse band located at a specific density between 1.064 and 1.074 g/ml. However the resulting parasites were shown to have low metabolic activity beyond 5 hours after purification and very low infectivity when used to initiate new cultures (Figueroa et al, 1990a). According to the authors, although in subsequent Western blotting analysis the free parasites were shown to retain antigenic characteristics, the exposure of iRBC to glycerol may have osmotically damaged the parasites and consequently decreased their ability to invade uninfected RBC.

Continuous gradient centrifugation has been also applied to concentrate free merozoites and iRBC of *B.bovis* (Rodriguez et al, 1986a). In that study it was reported that a density gradient generated from a 65% Percoll solution by centrifugation at 26,000 x g for 30 minutes allowed the best concentration of free merozoites, which had been obtained after CO_2 deprivation (Levy et al, 1981), in a diffuse band at a specific density of approximately 1.087 g/ml. The concentrated free merozoites proved to have 89% viability when used to initiate new cultures. In the same study the use of a 100% Percoll solution to generate density gradients was reported, using the same previously mentioned centrifugation conditions, which allowed concentration of infected erythrocytes at a specific density between 1.121 and 1.123 g/ml. The concentrated iRBC also proved to have a high capability for initiating new cultures (Rodriguez et al, 1986a).

Discontinuous gradient centrifugation under the conditions tested in the present study was disappointing with both *B.bovis* and *B.bigemina* parasites and did not result in appreciable concentration of iRBC. Although four density steps were used, covering a wide range of densities (from 1.050 to 1.121 g/ml), within which the iRBC were expected to concentrate, no concentration was achieved from *B.bigemina* infected blood, either after a centrifugation at 750 x g for 15 minutes or at 1,500 x g for 30 minutes (Fig 5.4). It appeared that either the g force of 750 was too low or that a longer period of time was required for separation, since the sample did not pass through the last two density steps and no interfaces were seen after the centrifugation. Similar conditions have been reported by Bhushan and co-workers to be suitable for separation of cultured equine RBC infected with *B.caballi* with parasitaemias between 0.4 and 1.5% (Bhushan et al, 1991). These authors used two density steps (1.080 and 1.115 g/ml) in a discontinuous Percoll gradient subjected to centrifugation at 500 x g for 20 minutes, which resulted in a band at the interface between the two steps that contained a high concentration of parasitized RBC (95-98%) and a large number of free merozoites, while most of the uninfected erythrocytes passed through both density steps and settled at the bottom of the tube.

Due to the lack of separation of *B.bigemina* infected blood after centrifugation at 750 x g for 15 minutes a second attempt was made in which the speed was increased to 1,500 x g and the length of time was increased to 30 minutes. However the separation was still incomplete and concentration of infected cells was not achieved, although one interface containing two bands was seen between the two most dense steps (1.095 and 1.121 g/ml). Therefore it was inferred that neither of the two discontinuous gradient conditions tested were suitable for concentrating *B.bigemina* iRBC from low parasitaemia blood.

The discontinuous density gradient centrifugation designed for concentration of *B.bovis* cultured RBC included three density steps which covered the range of densities in which separation of RBC was expected (1.060 to 1.121 g/ml). Nevertheless, the concentration achieved in one of the bands located in the interface above the most dense step (1.121 g/ml) resulted in an increase to only 8% from the parasitaemia of 5.5% estimated in the original culture suspension which was used for the separation. The concentration rate was 1.6 times, which it was considered poor. Therefore, as observed for *B.bigemina* infected blood, the discontinuous density centrifugation, under the conditions tested in the present study, did not seem to be a suitable method for concentration of *B.bovis* iRBC, particularly considering that this parasite could be successfully concentrated and partially purified from host cell contamination by other methods less expensive and easier to carry out such as differential hypotonic lysis using KCl (as described in section 3.6.1.1), or the induction of free merozoites by depriving cultures of CO_2, as reported in this chapter.

A comparison between the two methods used in the present study, regarding their efficiency in concentrating *B.bovis* parasites, indicated that the induction of free merozoites into the culture supernatant was more efficient than the use of density gradient centrifugation under the conditions used here. Nevertheless, for the purposes of this study, the differential hypotonic lysis using KCl still appeared to be a more appropriate method for production of bulked *B.bovis* antigenic preparations from cultures. Thus, it was the method used for provision of *B.bovis* extracts for the immunochemical analysis (Chapter 6) and production of crude ELISA antigen (Chapter 8).

In contrast, *B.bigemina* parasites are much more difficult to concentrate using conventional methods. In the present study, the continuous gradient centrifugation using a 68.46% Percoll solution subjected to centrifugation at 30,000 x g for 13 minutes produced high concentrations of parasitized RBC and proved to be a very efficient method for concentration of *B.bigemina* parasites from either infected blood or culture suspension. Thus, it was the method used for provision of concentrated *B.bigemina* material for the experiments on immunochemical characterisation (described in Chapter 6).

CHAPTER SIX

IMMUNOCHEMICAL CHARACTERISATION OF STOCKS OF *B.BIGEMINA* AND *B.BOVIS*

6.1 INTRODUCTION

Crude parasite preparations are commonly used as a source of antigen in immunodiagnostic assays. Due to the fact that *Babesia* parasites develop almost exclusively inside erythrocytes, such preparations frequently contain considerable amounts of host cell components which affect the performance of the assays by increasing the non-specific background. Attempts to improve specificity of immunodiagnostic assays for *Babesia* have therefore been directed at replacing conventional crude extracts with purified parasite-specific antigens (Bose et al, 1990).

A preliminary step towards achieving this is the characterisation of the parasite components present within crude preparations, using a number of biochemical methods such as gel filtration, one and two dimensional gel electrophoresis, affinity chromatography and isoelectric focusing. Subsequently, techniques such as immuno-blotting or immunoprecipitation are used to identify which of the parasite components are immunodominant antigens recognised by specific polyclonal antisera or monoclonal antibodies. These immunodominant antigens, if parasite-specific and isolate-common, have potential for purification and use in improved immuno-assays.

The experiments described in this chapter were designed with the aim of characterising immunochemically both exoantigens and somatic components of geographically different stocks of *B.bovis* and *B.bigemina* in order to identify candidate proteins for use as specific diagnostic tools.

The analysis of somatic components included a preliminary study in which *B.bovis* and *B.bigemina* iRBC antigens were prepared with and without the use of enzyme inhibitors to determine the most suitable method for preparation of samples to be used in SDS-PAGE and immuno-blotting analysis. Using somatic component preparations, a panel of eleven *B.bovis* monoclonal antibodies was characterised by IFAT, ELISA and immuno-blotting analysis. This panel of monoclonal antibodies was used as a means to identify *B.bovis* specific antigens and to see whether diversity exists among *B.bovis* isolates.

Polyclonal and monoclonal antibodies were then both used to identify immunodominant components present in different *Babesia* lysates by immuno-blotting. The polyclonal antibodies were obtained from a panel of calf sera raised experimentally against the different *Babesia* stocks, and a panel of serum samples taken from cattle naturally exposed to tick infestation in the field in Brazil.

In order to analyse exoantigens, culture supernatants of both *Babesia* parasites were concentrated and fractionated by high performance liquid chromatography (HPLC), first by size exclusion fractionation, and followed by ion exchange fractionation. These exoantigens were analysed using immuno-blotting techniques with the polyclonal sera described above.

6.2 STUDIES ON ANTIGEN PREPARATION

6.2.1 Materials and methods

Parasite extracts of *B.bovis* and *B.bigemina* were obtained from *in vitro* culture suspensions (see section 3.3), and SDS-PAGE and immuno-blotting were performed as described previously (see sections 3.6 and 3.7).

For this study, *B.bovis* iRBC (Kwanyanga stock) were prepared as described in section 3.6.1.1 and 200 µl aliquots of the resultant pellet were used to prepare *B.bovis* antigens (designated bo).

B.bigemina iRBC (Mexico stock) were prepared as described in Chapter 5 (section 5.3.1) and 200 µl aliquots of pellet were used to prepare *B.bigemina* antigens (designated bi).

Aliquots of bo and bi were treated as follows:

Sample	**Treatment with enzyme inhibitors***
bo 1 and bi 1	addition of 200 µl PBS containing 5 mM EDTA, 0.2 mM leucopeptin and 1 mM PMSF
bo 2 and bi 2	lysis with 400 µl of TBS buffer containing 5 mM iodoacetamide, 2 mM PMSF, 5 mM EDTA, 2 mM sodium metabisulphite, 0.1 mM TLCK, 1% NP-40 and 1% sodium deoxycholate
bo 3 and bi 3	none

* see list of abbreviations on page viii.

One volume of each sample (200 µl) was then mixed with an equal volume of SDS-reducing sample buffer (see Appendix 1), boiled for 5 minutes and centrifuged at 10,000 x g for 3 minutes. Supernatants were subjected to SDS-PAGE in 10% homogeneous gels. A sample of molecular weight markers (ranging from 21.5 to 200 kDa) was included in all gels. After electrophoresis, separated proteins were transferred to nitrocellulose membranes by Western immuno-blotting. For *B.bovis* samples, the resulting blot was probed with a pool of *B.bovis* antisera (from calves 396 and P78, both taken on day 69 after infection). For *B.bigemina* samples the blot was probed with a pool of *B.bigemina* antisera (from calves 397, 399 and 583, taken on days 69, 98 and 85 respectively).

6.2.2 Results

Homologous sera detected antigens in all three preparations of both *B.bovis* and *B.bigemina* samples, as shown in Fig 6.1.

A maximum of 11 antigenic bands were detected by homologous sera in *B.bovis* samples (bo 3). The major antigen in all three sample preparations was a protein of molecular weight 56 kDa. The greatest number of bands and the strongest reactions were detected in preparation bo 3, followed by preparation bo 1 and the weakest bands were detected in preparation bo 2, as shown in Fig 6.1 A.

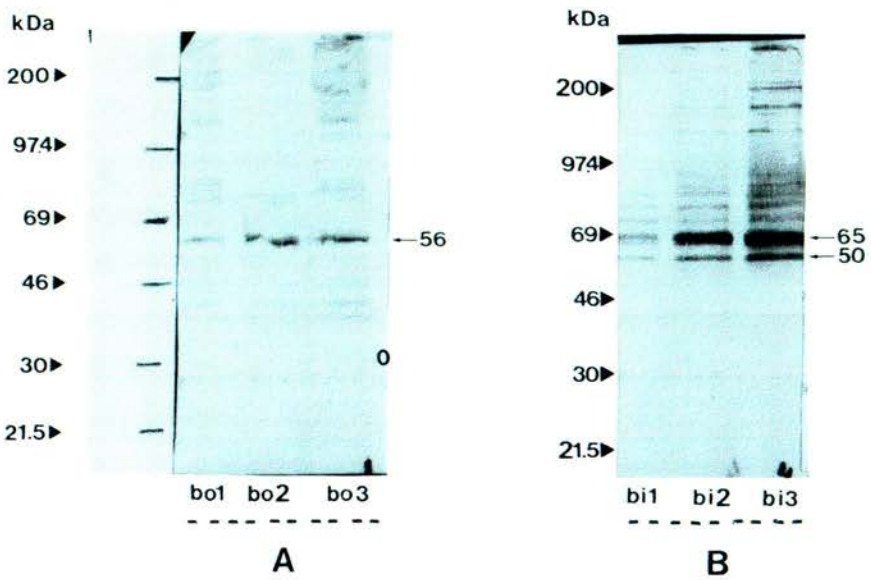
A maximum of 16 bands were detected by homologous sera in *B.bigemina* samples (bi 3). The major antigens detected in all three preparations were proteins with molecular weights 65 and 50 kDa. The greatest number of bands and the strongest reactions were detected in preparation bi 3, followed by preparation bi 2 and bi 1, as shown in Fig 6.1 B.

It was concluded that for both *B.bovis* and *B.bigemina*, lysis of pelleted parasites with SDS sample buffer without enzyme inhibitors was the most suitable method for preparation of samples for use in the SDS-PAGE and immuno-blotting analysis described in this chapter.

Fig 6.1 Western immuno-blotting analysis of *B.bovis* (Kwanyanga stock) and *B.bigemina* (Mexico stock) antigens prepared with or without enzyme inhibitors.

Blot A (lanes bo 1-3) contains *B.bovis* samples tested against a pool of anti-*B.bovis* sera.
Blot B (lanes bi 1-3) contains *B.bigemina* samples tested against a pool of anti-*B.bigemina* sera.
Standard molecular weight markers are indicated on the left side.
Arrows on the right indicate immunodominant proteins.

Fig 6.1


kDa
200
974
69
46
30
21.5
56
0
bo1
bo2
bo3
A
kDa
200
974
69
46
30
21.5
65
50
bi1
bi2
bi3
B

6.3 CHARACTERISATION OF MONOCLONAL ANTIBODIES

6.3.1 Materials and methods

The panel of eleven McAbs raised against the Kwanyanga stock of *B.bovis* at the Wellcome Laboratories (as described in section 3.4.3) was characterised by IFAT, ELISA and Western immuno-blotting.

6.3.1.1 IFAT

The IFAT was carried out as described in section 3.5 except that cytospin smears were used as antigen instead of plain smears. This was to give better visualisation of parasites allowing the specific reactivity of an antibody against a particular component of the parasite or infected cell to be observed. Cytospin smears (two per slide) were prepared from each stock of *B.bovis* and tested against each McAb diluted 1 in 100 in PBS. Plain antigen smears of *B.bigemina* (Zaria and Muguga stocks) were included in the study to evaluate the species-specificity of the McAbs. Sera from calf 396 (see Table 3.1), taken on days 0 and 69 after *B.bovis* infection, diluted 1 in 160 with PBS, were used as negative and positive controls, respectively. A PBS control was also included on each slide.

6.3.1.2 ELISA

The ELISA was carried out using a crude *B.bovis* lysate from a culture suspension of the Lismore stock as antigen (Woodford et al, 1990). Each McAb was titrated by serial doubling dilutions from 1 in 25 to 1 in 3,200. The assay was performed as described in section 3.8; a McAb raised against *Trypanosoma evansi* (McAb 2G2, IgG 1, Frame et al, 1990) was used as a negative control. Absorbance values higher than the cut-off point 0.2, based on the OD of the negative control, were considered positive.

6.3.1.3 Immuno-blotting

For Western immuno-blotting analysis, four pools of McAbs were tested against three stocks of *B.bovis*. Pool A consisted of McAbs 4F3:1, 1C12:1 and 2C8:2; pool B consisted of McAbs 118.672, 4D1:3 and 3D6:1; pool C consisted of McAbs 3D42 and 1C12:2; and pool D consisted of McAbs 5G12 and 1B8:1. Each pool was tested at a dilution of 1 in 100. The anti-*T.evansi* McAb 2G2 was also used as a negative control in Western immuno-blotting.

6.3.2 Results

6.3.2.1 IFAT

The results from the IFAT analysis are illustrated in Table 6.1 and show that, with one exception, all *B.bovis* McAbs reacted with all the stocks of *B.bovis* parasites; only McAb 2A11 failed to react with the Lismore stock of *B.bovis*. For the other McAbs the strongest reactions were seen with the Lismore antigen.

The eleven McAbs were classified into three groups according to their pattern of reactivity by IFAT. Group 1 includes those McAbs which recognised internal parasite components (5G12, 1B8:1, 4F3:1, 3D6:1 and 2C8:2), group 2 included the McAbs which recognised parasite and infected RBC cytoplasm/membrane components (1C12:1, 4D1:3, 118.672 and 2A11), and group 3 included the McAbs which reacted with the parasite surface (3D42 and 1C12:2).

In general the pattern of reactions of individual McAbs was similar for the three *B.bovis* antigens, only the intensity of reactions differed between the stocks, as shown in Table 6.1. The intensity (brightness) of reactions also varied between McAbs with 3D42, 1C12:2, 3D6:1 and 4D1:3 giving brightest reactions.

No McAbs showed cross-reactivity with any of the *B.bigemina* parasites. However McAbs 1B8:1, 4F3:1 and 2C8:2 (group 1), and 3D42 (group 3) reacted with erythrocyte components (membrane or cytoplasm) of some RBC infected with *B.bigemina* of both Zaria and Muguga stocks.

No reactions were seen with the negative controls (pre-infection and PBS).

6.3.2.2 ELISA analysis

Only four of the eleven McAbs reacted in ELISA with the *B.bovis* (Lismore) antigen. These were 4F3:1 (end point titre of 1 in 400), 1B8:1 (end point titre 1 in 200), 2A11 and 118.672 (both with end point titres of 1 in 25).

Table 6.1 IFAT staining patterns of a panel of eleven *B.bovis* McAb tested against *B.bovis* (Lismore, Kwanyanga and Mexico) antigens.

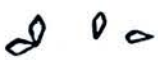
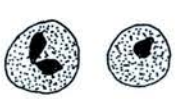
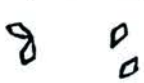
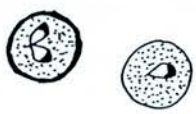
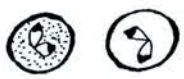
McAb (class)*/staining pattern	Antigen Lismore	Kwanyanga	Mexico
5G12 (IgG 2b) 	++ parasites (free + intracellular); dot in RBC	+ faint parasites, faint cytoplasm in few iRBC; small dot in RBC	+ faint parasites; small dot in RBC
1B8:1 (not known) 	+ faint parasite	+ faint parasite; faint cytoplasm in few iRBC	+ faint parasite (free merozoites brighter); faint cytoplasm in some iRBC
4F3:1 (IgG) 	++ parasite (free + intracellular)	++ parasite (free + intracellular)	+ faint intracellular parasite; few free merozoites
3D42 (IgG 2a) 	+++ parasite surface (free + intracellular)	+++ parasite surface (free + intracellular); faint cytoplasm in few iRBC	++ free merozoite surface; faint cytoplasm of iRBC (diffuse)
1C12:2 (not known) 	+++ parasite surface (free + intracellular)	+++ parasite surface (free stronger than intracellular); faint cytoplasm of iRBC (diffuse)	+++ parasite surface (free + intracellular)
3D6:1 (IgG 2b) 	+++ only part of the parasite	++ only part of the parasite	++ small part of the parasite; cytoplasm of few iRBC
1C12:1 (IgG 2a) 	++ parasite; cytoplasm of iRBC	+ some free merozoites; iRBC cytoplasm	+ parasite (free + intracellular); iRBC cytoplasm
2C8:2 (not known) 	++ few parasites	++ surface of some free merozoite; few intracellular parasites	+ surface of few free merozoite; some iRBC (diffuse)
2A11 (not known) 	--- no reaction	+ faint surface of free merozoite; faint few iRBC	+ faint iRBC (parasite + cytoplasm)

Table 6.1 Continuation

McAb (class)*/staining pattern	Antigen Lismore	Kwanyanga	Mexico
4D1:3 (IgG) 	++ parasite (extremity); iRBC membrane	++ parasite extremity (free + intracellular); cytoplasm and membrane of iRBC	+++ parasite extremity (free + intracellular); cytoplasm and membrane of some iRBC
118.672 (IgG 2a) 	++ RBC membrane; cytoplasm of some iRBC; faint parasite extremity	++ RBC membrane; cytoplasm of few iRBC	+ RBC membrane; cytoplasm of iRBC; faint parasite extremity

Key: +++ very bright
++ bright
+ faint
— not at all

RBC uninfected red blood cells
iRBC infected red blood cells

* S.Crowe, personal communication

6.3.2.3 Immuno-blotting analysis

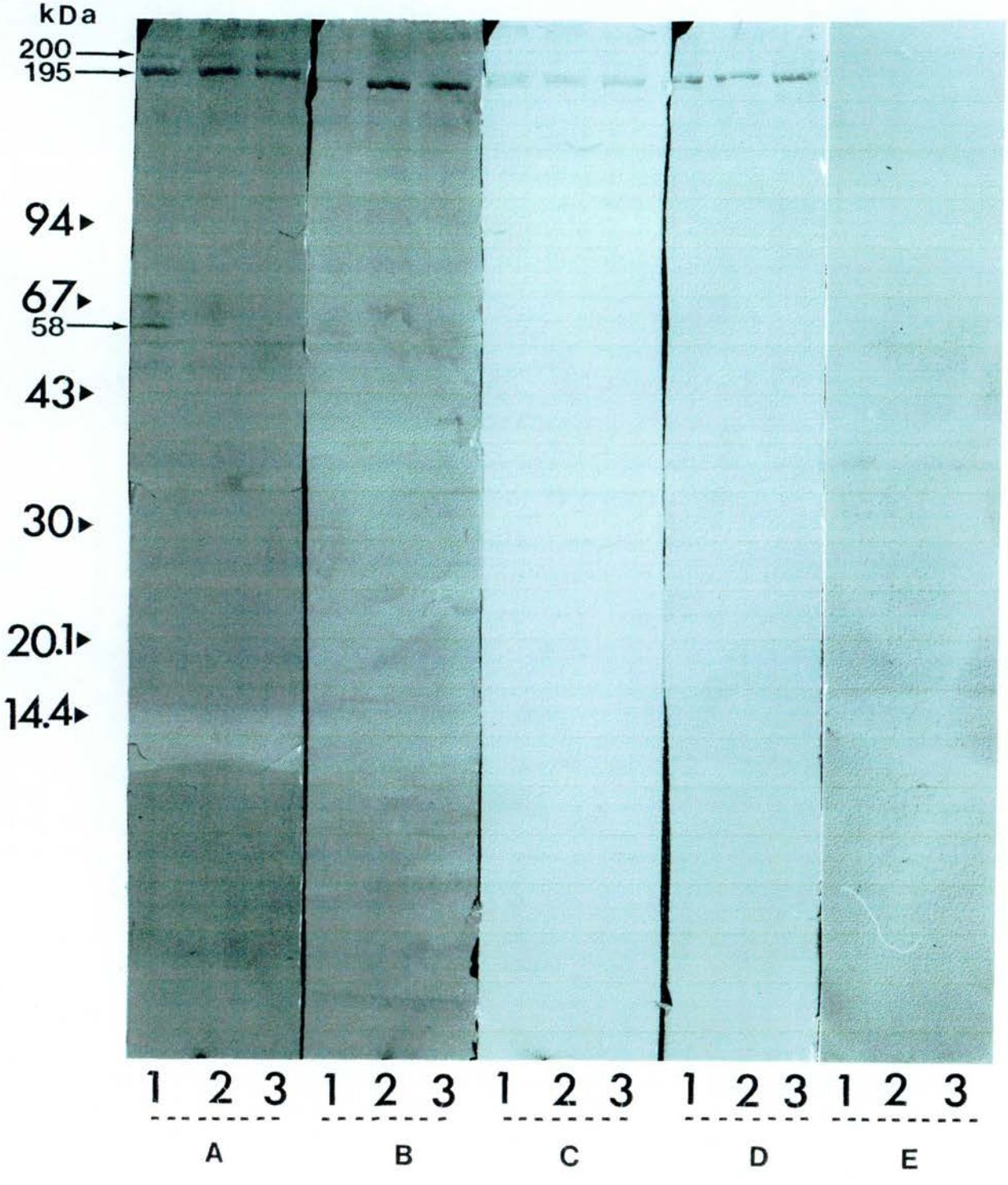
A 195 kDa antigen was detected in all three *B.bovis* stocks by the four McAb pools, as shown in Fig 6.2. Differences in staining intensity of this antigen were observed between the pools. The antigen was recognised strongly by pools A, B and D but hardly at all by pool C.

McAb pool A also detected a 200 kDa antigen in all the three *B.bovis* stocks and a 58 kDa antigen present only in the Kwanyanga stock of *B.bovis* (Fig 6.2 panel A).

No reaction was observed with the negative control anti-*Trypanosoma* McAb (Fig 6.2, panel E).

Fig 6.2 Western immuno-blotting of *B.bovis* antigens probed with pools of McAbs.
Lane 1 is Kwanyanga stock; lane 2 is Mexico stock; and lane 3 is Lismore stock.
A is a pool of McAbs 4F3:1, 1C12:1 and 2C8:2
B is a pool of McAbs 118.672, 4D1:3 and 3D6:1
C is a pool of McAbs 3D42 and 1C12:2
D is a pool of McAbs 5G12 and 1B8:1
E is an anti-*Trypanosoma evansi* McAb
Standard molecular weight markers are indicated on the left side.

Fig 6.2



6.4 ANALYSIS OF SOMATIC COMPONENTS OF *B.BOVIS*

6.4.1 Materials and methods

Samples of each *B.bovis* stock (Lismore, Mexico and Kwanyanga) were prepared as described in section 3.6.1.1. The final pellet containing concentrated iRBC was mixed with 2 volumes of SDS-sample buffer (Appendix 1) and then separated by SDS-PAGE in either homogeneous or gradient acrylamide gels (see section 3.6.2). A pellet of uninfected RBC was included as a negative control. Protein bands were stained with Coomassie blue (as described in section 3.6.3).

Western immuno-blotting was carried out as described in section 3.7 using homologous anti-*B.bovis* serum samples (calves 396, P78, 198, P49 and C11) and heterologous anti-*B.bigemina* serum samples (calves 397, 399, 583 and 8T), taken before and at different times after infection. Calf infections from which sera were used are detailed in Table 3.1. A pool of 6 serum samples collected from adult cattle which had been naturally exposed to tick infestation in Brazil (field sera) was also tested against each of the *B.bovis* stocks.

A blot of uninfected RBC lysate was probed with a pool of anti-*B.bovis* sera as a negative control.

6.4.2 Results

6.4.2.1 Total protein profiles

Protein patterns of Coomassie blue stained gels containing the three stocks of *B.bovis* and the uninfected RBC sample are shown in Fig 6.3. At least 42 bands ranging from 200 to 14 kDa were detected by Coomassie blue. There were no detectable differences between the protein profiles of the three stocks of *B.bovis* and the uninfected RBC preparation.

Fig 6.3 Coomassie blue staining of a 5-15% gradient gel containing samples of *B.bovis* and uninfected RBC.

Lanes 1 Lismore stock

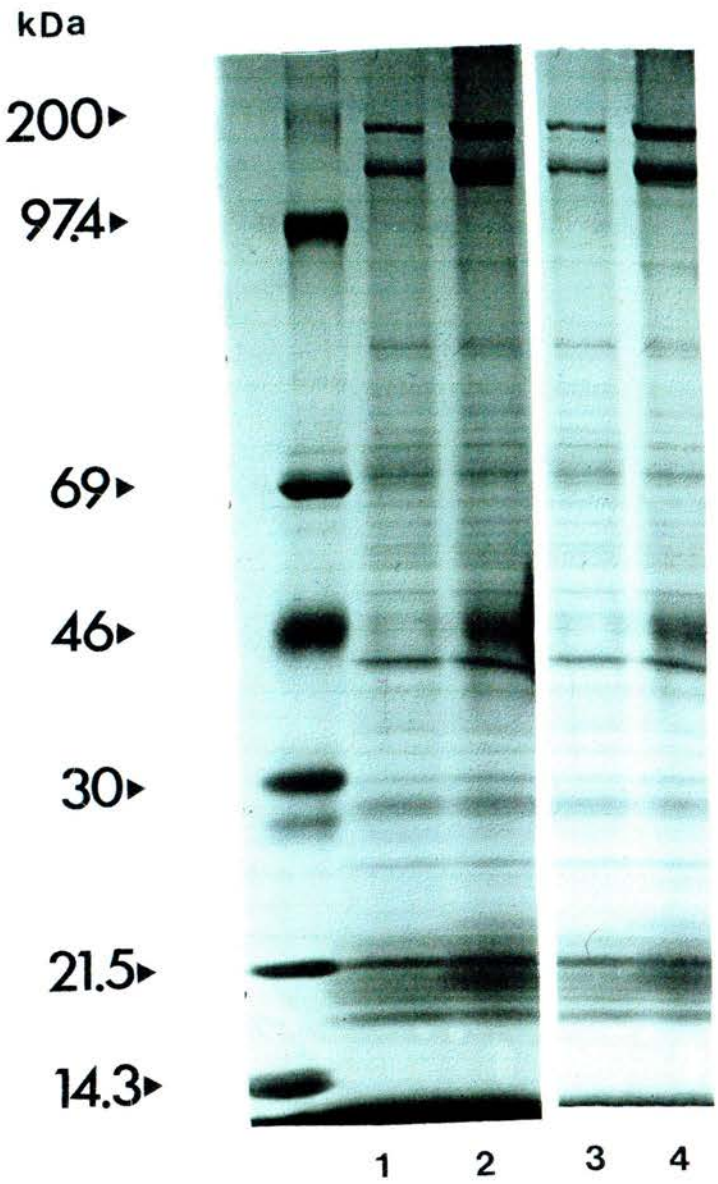
2 Mexico stock

3 Kwanyanga

4 uninfected RBC lysate.

Standard molecular weight markers are indicated on the left side.

Fig 6.3



6.4.2.2 Antigen profile

Several antigenic bands were recognised by sera raised against *B.bovis* stocks (Figs 6.4 -6.6).

Anti-Kwanyanga sera (from calves 198 and C11, both taken on day 28 after infection) recognised the same number of antigenic proteins (9) in both Kwanyanga and Mexico stocks but with different intensities (Fig 6.4 A). Fewer bands and less intensive reactions were observed with the Lismore stock.

A pool of sera from calf P78, taken after it had been challenged twice each with Kwanyanga and Lismore stocks, recognised approximately 20 antigenic proteins, which showed similar molecular weights and staining intensities in both Kwanyanga and Mexico stocks (Fig 6.4 B). In the Lismore stock some bands were detected which were absent in the other two stocks. These occurred at molecular weights of 30, 78 and 195 kDa. In addition, some proteins which were strongly recognised in the Kwanyanga and Mexico stocks, were not detected in the Lismore stock. These occurred at molecular weights of 70, 68, and 37-39 kDa (Fig 6.4 B) and corresponded to the bands which were absent in the Lismore stock when probed with anti-Kwanyanga sera.

The homologous system (anti-Mexico serum against the Mexico stock) showed that the number of bands increased from 5 (day 28) to 13 (day 104) (Fig 6.5, lanes 2, 5, 8 and 11). The strongest antigenic bands occurred at molecular weights 37 and 56 kDa and, later in the infection, at 140 kDa. Although most bands increased in intensity during the infection, there were still only two major antigens. A similar result was obtained with the heterologous system anti-Mexico sera/Kwanyanga stock (Fig 6.5, lanes 1, 4, 7 and 10). The heterologous system anti-Mexico sera/Lismore stock showed only one major antigen at 56 kDa throughout the infection (Fig 6.5, lanes 3, 6, 9 and 12). In contrast to the Kwanyanga and Mexico stocks, the Lismore stock did not show antigenic bands at molecular weights 37-39, and only a very faint reaction was seen against a 140 kDa antigen even 104 days after infection.

Fig 6.6 A shows that the pool of Brazilian field sera reacted more strongly against antigens in the Kwanyanga stock than in the other two stocks. Major reactions were detected at molecular weights 200, 140 and 56 kDa. The 200 and 56 kDa were also the major antigens detected in the Mexico and Lismore stocks. The Brazilian field sera recognised a 37-39 kDa protein in the Kwanyanga and Mexico stocks but not in the Lismore stock. However only in the latter stock was a 30 kDa antigen detected. A minor antigen at 31 kDa was faintly detected in the Kwanyanga and Mexico stocks.

The pool of anti-*B.bigemina* sera showed faint reactivity against four antigens present in all three samples of *B.bovis*. These bands were seen at molecular weights 200, 72, 49 and 23 kDa, as shown in Fig 6.7 C (arrows). These cross-reactive bands corresponded to bands

detected by anti-*B.bovis* sera as shown in Fig 6.7 A and B. However they were not the major antigenic *B.bovis* bands.

Only two very faint bands (at 58 and 49 kDa) were detected when the uninfected RBC preparation was probed with the pool of anti-*B.bovis* sera, which included sera raised against all three stocks (Fig 6.6 B).

The pool of pre-infection sera did not react against the major antigenic bands of *B.bovis* samples. However a faint reaction against a band of 200 kDa was detected with this pool (Fig 6.6 C, arrow).

A summary of the *B.bovis* antigens detected by immuno-blotting is presented in Table 6.2.

Fig 6.4 Western immuno-blotting analysis of *B.bovis* antigens probed with stock-specific antisera.

Lanes 1 and 4 are Kwanyanga stock, lanes 2 and 5 are Mexico stock, and lanes 3 and 6 are Lismore stock.

In A a pool of sera anti-Kwanyanga stock, taken on day 28 after infection was used.

In B a pool of sera from calf P78 infected with Lismore stock and challenged twice with Lismore and Kwanyanga stocks was used.

Standard molecular weight markers are indicated on the left side.

Fig 6.4

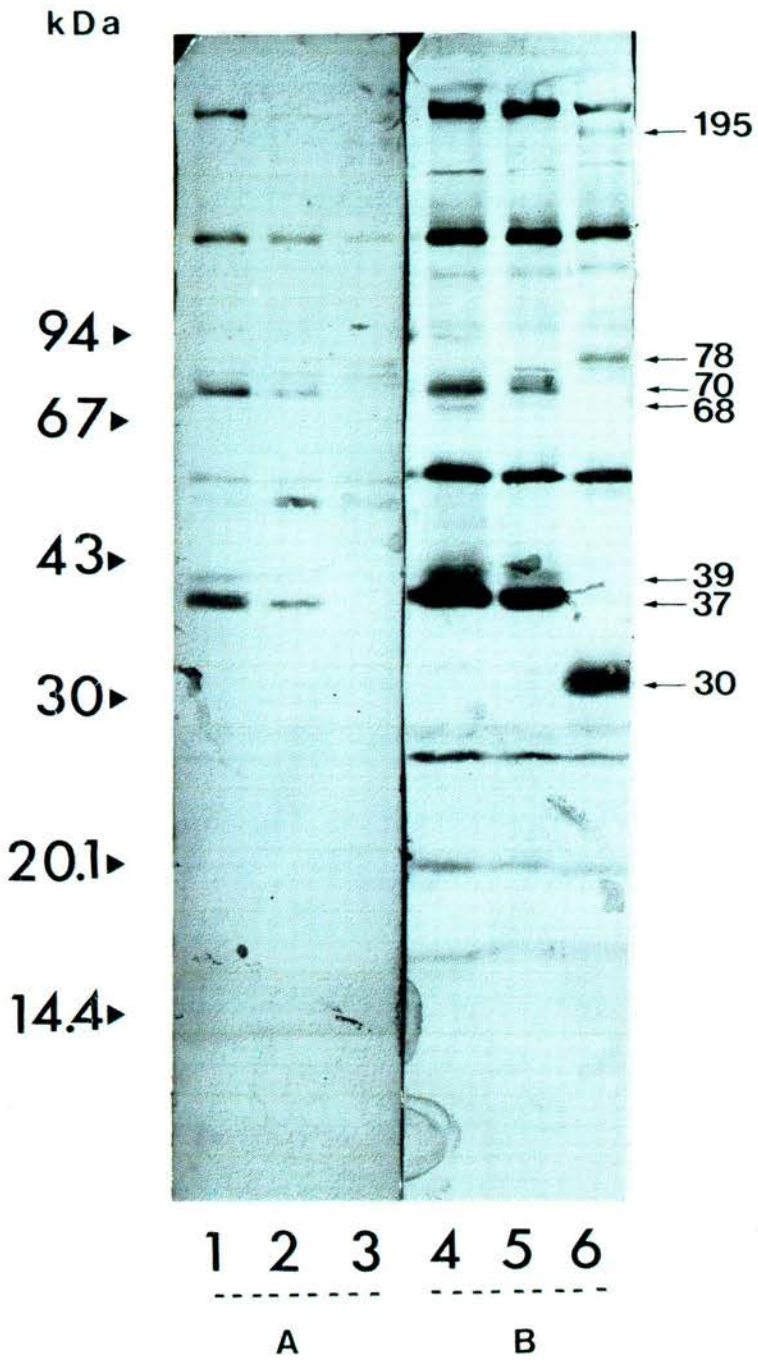


Fig 6.5 Western immuno-blotting analysis of *B.bovis* antigens probed with anti-Mexico stock sera (from calf 396).

Lanes 1, 4, 7 and 10 are Kwanyanga stock; lanes 2, 5, 8 and 11 are Mexico stock; lanes 3, 6, 9 and 12 are Lismore stock, probed with:

A - serum taken on day 28 post-infection (p.i.)

B - serum taken on day 49 p.i. (21 days after the first challenge)

C - serum taken on day 77 p.i. (21 days after the second challenge)

D - serum taken on day 104 p.i. (14 days after the third challenge).

Standard molecular weight markers are indicated on the left side.

Fig 6.5

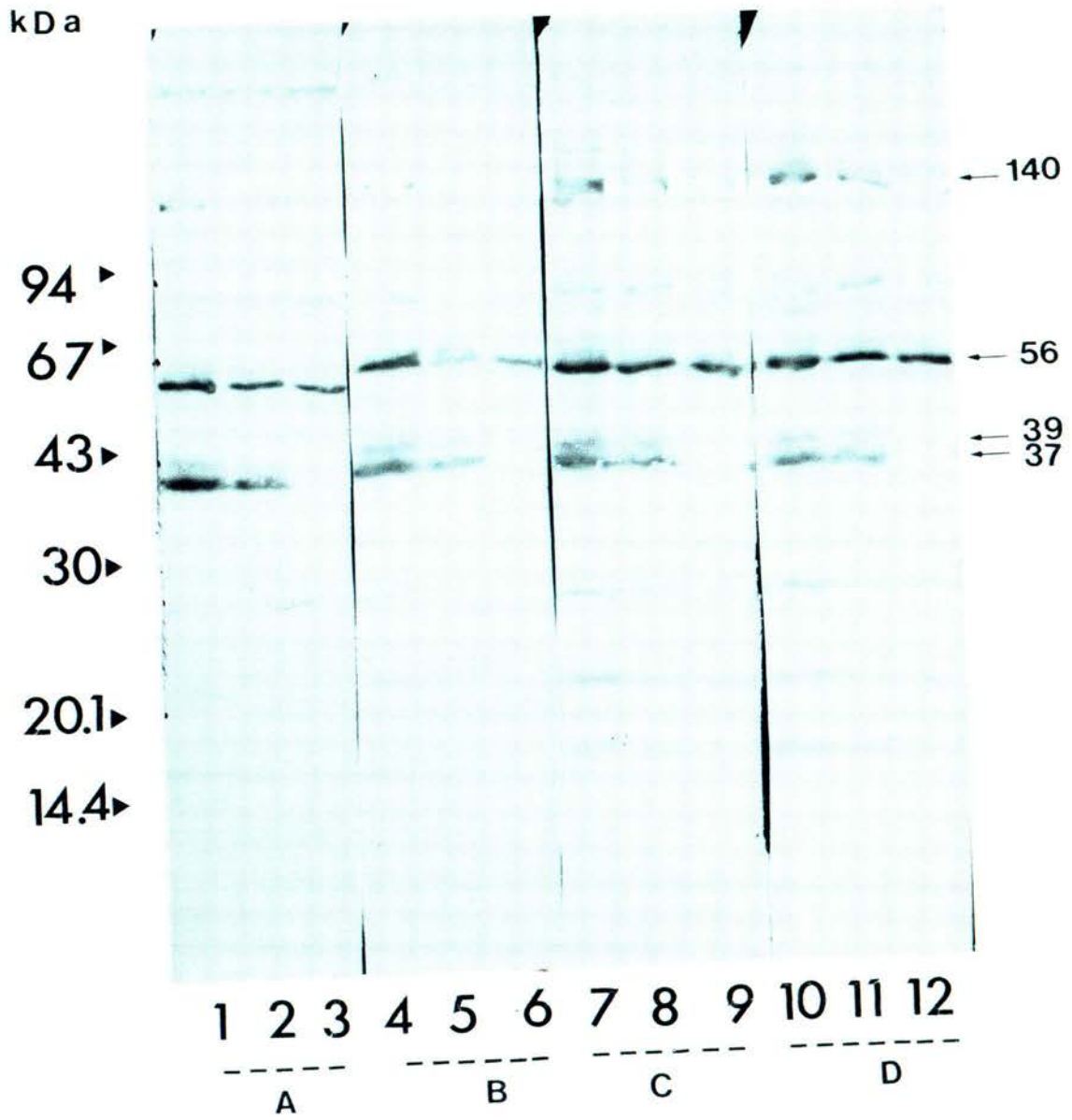


Fig 6.6 Western immuno-blotting analysis of *B.bovis* and uninfected RBC antigens.
Lane 1 is Kwanyanga stock; lane 2 is Lismore stock; lane 3 is Mexico stock; and lane 4 is an uninfected RBC lysate.
A was probed with a pool of field sera from Brazil; B was probed with a pool of anti-*B.bovis* sera; and C was probed with a pool of pre-infection sera.
Standard molecular markers are indicated on the left.

Fig 6.6

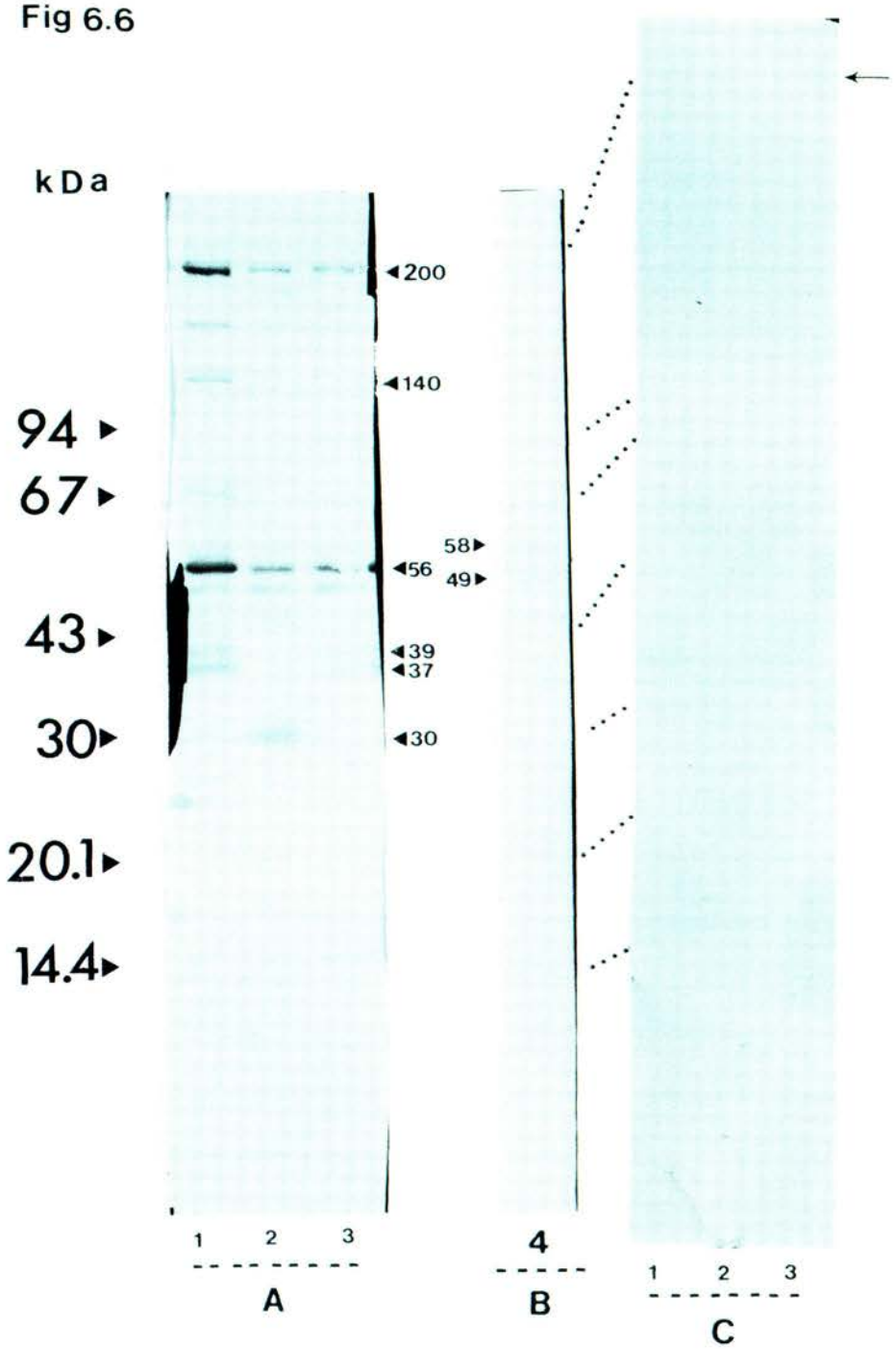


Fig 6.7 Western immuno-blotting of *B.bovis* antigens probed with a pool of anti-*B.bovis* (Lismore) sera (A); a pool of anti-*B.bovis* (Mexico) sera (B); and a pool of anti-*B.bigemina* (Zaria) sera (C).

Lane 1 is Kwanyanga stock, lane 2 is Lismore and lane 3 is Mexico stocks.

Standard molecular weight markers are indicated on the left-hand side.

Faint cross-reactive bands are indicated on the right side.

Fig6.7

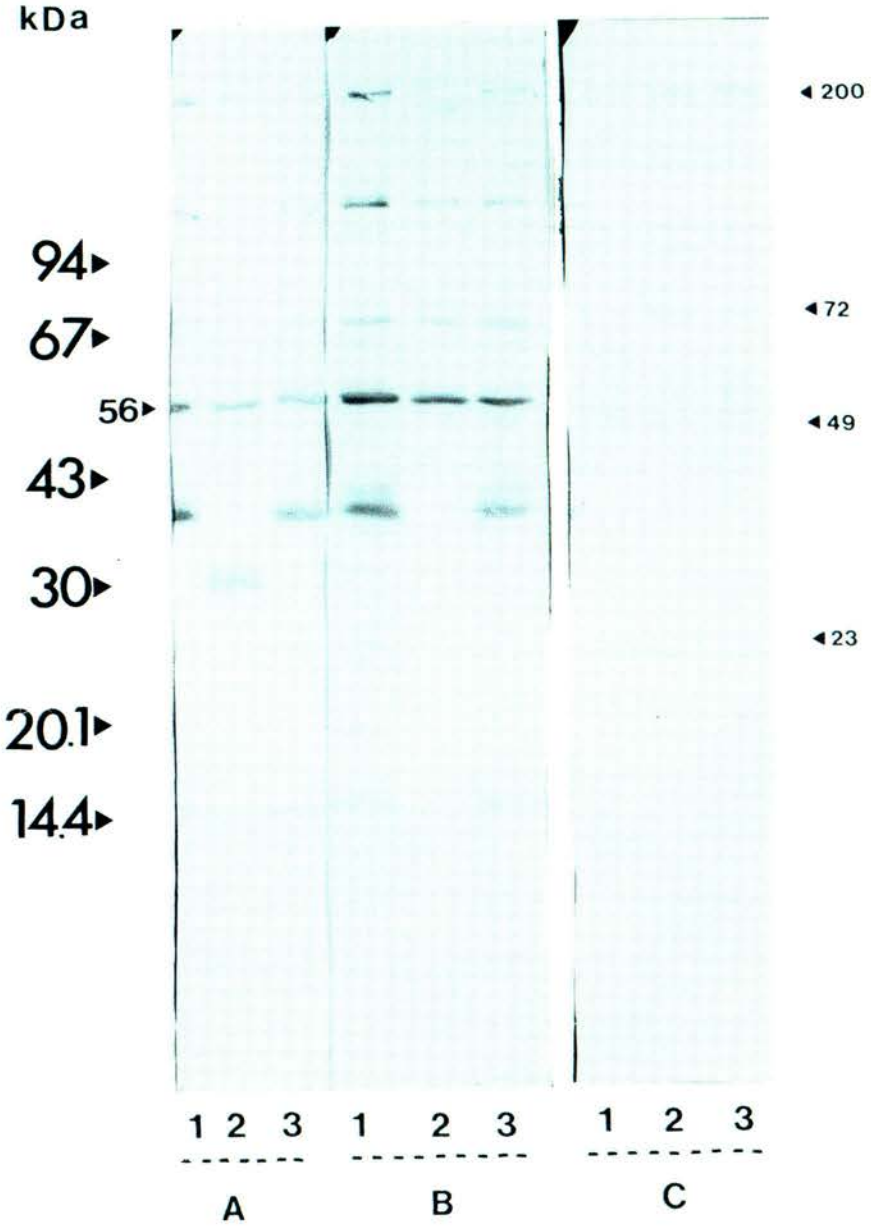


Table 6.2 Summary of protein bands of *B.bovis* antigens detected by Western immuno-blotting.

Antigen molecular weight (kDa)	Sera											
	anti-*B.bovis*			field sera			anti-*B.bigemina*			pre-infection		
	L	M	K	L	M	K	L	M	K	L	M	K
200	—	—	—	—	—	—	—	—	—	—	—	—
195	—			—								
185	—	—	—	—		—						
140	—	—	—			—						
121	—	—	—									
95	—	—	—									
78	—											
76	—	—	—			—						
72	—	—	—			—	—	—	—			
70		—	—									
56	—	—	—	—	—	—						
49	—	—	—	—	—	—	—	—	—			
39		—	—	—	—	—						
37		—	—	—	—	—						
31		----	----		—	—						
30	—			—								
28	—	—	—									
27	—	—	—									
23	—	—	—				—	—	—			
18	—	—	—									
14	—	—	—	—	—	—						

Key: L - Lismore stock
M - Mexico stock
K - Kwanyanga stock

6.5 ANALYSIS OF SOMATIC COMPONENTS OF *B.BIGEMINA*

6.5.1 Materials and methods

Two stocks of *B.bigemina* were included in this study, Mexico and Kenya. The sample of the Kenyan stock was obtained from the International Laboratory for Research on Animal Diseases (ILRAD) (Kenya) and had been prepared from infected bovine blood as follows (J.Katende, personal communication). Blood was collected in Alsever's solution and blood cells were washed three times with PBS by centrifugation at 1,500 x g for 30 minutes at 4°C. The buffy coat was removed after each centrifugation and the resulting pellet from the last centrifugation was added to an equal volume of a 20% solution of DMSO in PBS. The mixture was aliquoted into 250 ml volumes and stored in liquid nitrogen. One aliquot was thawed in a water bath at 56°C, sonicated (40-50 relative output) for 1-2 minutes and centrifuged at 200 x g for 30 minutes at 4°C. The supernatant which contained most of the merozoites was centrifuged at 500 x g for 30 minutes and pelleted merozoites were washed three times with PBS/glucose (pH 8.0). The isolated merozoites were suspended in 2 ml of PBS/glucose containing 0.2 M NaCl and protease inhibitors, and disrupted by sonication for 1 minute (relative output 80) on ice. The lysate was centrifuged at 4,500 x g for 30 minutes to pellet any insoluble material and the supernatant was stored at -80°C as 100 µl aliquots and sent to CTVM.

When required, one aliquot was thawed out and mixed with 2 volumes of SDS-PAGE reducing buffer (Appendix 1). The suspension was boiled for 5 minutes and then centrifuged at 10,000 x g for 3 minutes. The resulting supernatant of *B.bigemina* (Kenya) was used for SDS gradient gel electrophoresis.

The Mexico stock of *B.bigemina* from an *in vitro* cultured RBC suspension was prepared as described in Chapter 5. Concentrated *B.bigemina* iRBC obtained by this method were then washed three times by centrifugation with PBS. Two volumes of SDS reducing buffer sample were added to the final pellet; the suspension was boiled and centrifuged as described above. The resulting supernatant of *B.bigemina* (Mexico) was used for SDS gel electrophoresis on 10% homogeneous acrylamide gels (as described in section 3.6.2).

Protein bands were stained with Coomassie blue (as described in section 3.6.3). Parasite antigens were identified using serum samples from calves 8T, 397, 583 and 399 (see Table 3.1), which were used either individually or as a pool. *B.bigemina* antigens were also tested against a pool of anti-*B.bovis* sera (from calves 396, P78 and 198) to investigate cross-reactivity. A pool of pre-infection sera was included as a negative control. A lysate of uninfected RBC was probed with the pool of anti-*B.bigemina* sera.

6.5.2 Results

6.5.2.1 Total protein profiles

The *B.bigemina* (Kenya) total protein profile is presented in Fig 6.8 A. Approximately 30 protein bands were detected with molecular weights ranging from 200 to 14 kDa. Fig 6.8 B shows the total protein profile of the Mexico stock of *B.bigemina* and, although poor staining did not allow accurate quantification of the *B.bigemina* proteins, several groups of proteins were detected in the molecular weight range 200 to 14 kDa.

6.5.2.2 Antigen profiles

a. Kenya stock

Fig 6.9 shows the antigenic bands of *B.bigemina* (Kenya stock) obtained with individual serum samples taken at different times after infection with *B.bigemina*. Serum from calves 397 and 583 taken respectively on days 89 and 97 post-infection with Zaria stock showed the greater number of antigens (13) and the highest staining intensity (Fig 6.9, lanes 2 and 3). The antigens were seen at molecular weights ranging from 100 to 40 kDa. All sera, except 8T, recognised two major antigens at 42 and 60 kDa. Serum from calf 8T recognised two faint antigens at 45 and 67 kDa, one of which (45 kDa) was also recognised by serum from calf 583.

The pool of pre-infection sera did not react with any of the *B.bigemina* bands (Fig 6.9, lane 8).

The pool of anti-*B.bovis* sera reacted faintly against a band of molecular weight 65kDa (Fig 6.9, lane 9).

b. Mexico stock

Approximately 15 bands, molecular weights ranging from 250 to 50 kDa, were detected in *B.bigemina* (Mexico stock) by anti-*B.bigemina* sera (Fig 6.10 A). The major antigens (strongest reactions) were seen at molecular weights 65 and 50 kDa. A faint reaction was detected against two bands at 69 and 71 kDa with both the pool of pre-infection sera (Fig 6.10 B) and the pool of anti-*B.bovis* sera (Fig 6.10 C).

A summary of the *B.bigemina* antigens detected by immuno-blotting is presented in Table 6.3.

Fig 6.8 Coomassie blue staining of acrylamide gels showing total protein profiles of *B.bigemina.*

Lane 1 is a sample of the Kenya stock separated in a 7-20% gradient gel.

Lane 2 is a sample of the Mexico stock separated in a 10% homogeneous gel.

Standard molecular weight markers are indicated on the left side.

Fig 6.8

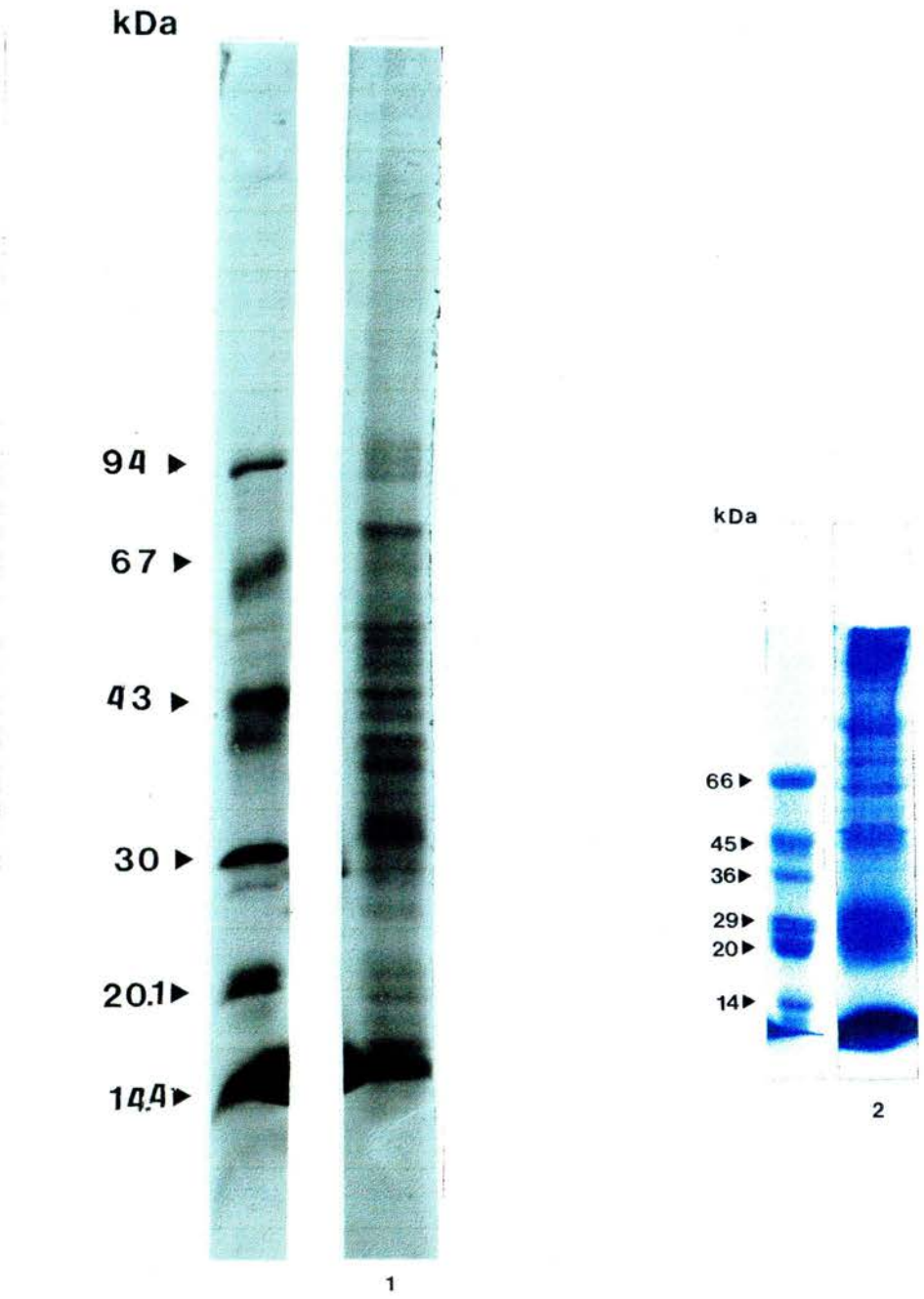


Fig 6.9 Western immuno-blotting of *B.bigemina* (Kenya stock) antigens probed with calf sera taken before and at different times after *B.bigemina* infection:

Lanes 1 - serum from calf 8T taken on day 28 post-infection (pi)
2 - serum from calf 397 taken on day 89 pi
3 - serum from calf 583 taken on day 97 pi
4 - serum from calf 399 taken on day 29 pi
5 - serum from calf 399 taken on day 50 pi
6 - serum from calf 399 taken on day 71 pi
7 - serum from calf 399 taken on day 119 pi
8 - a pool of pre-infection sera
9 - a pool of anti-*B.bovis* sera

Standard molecular weight markers are indicated on the left side.

Fig 6.9

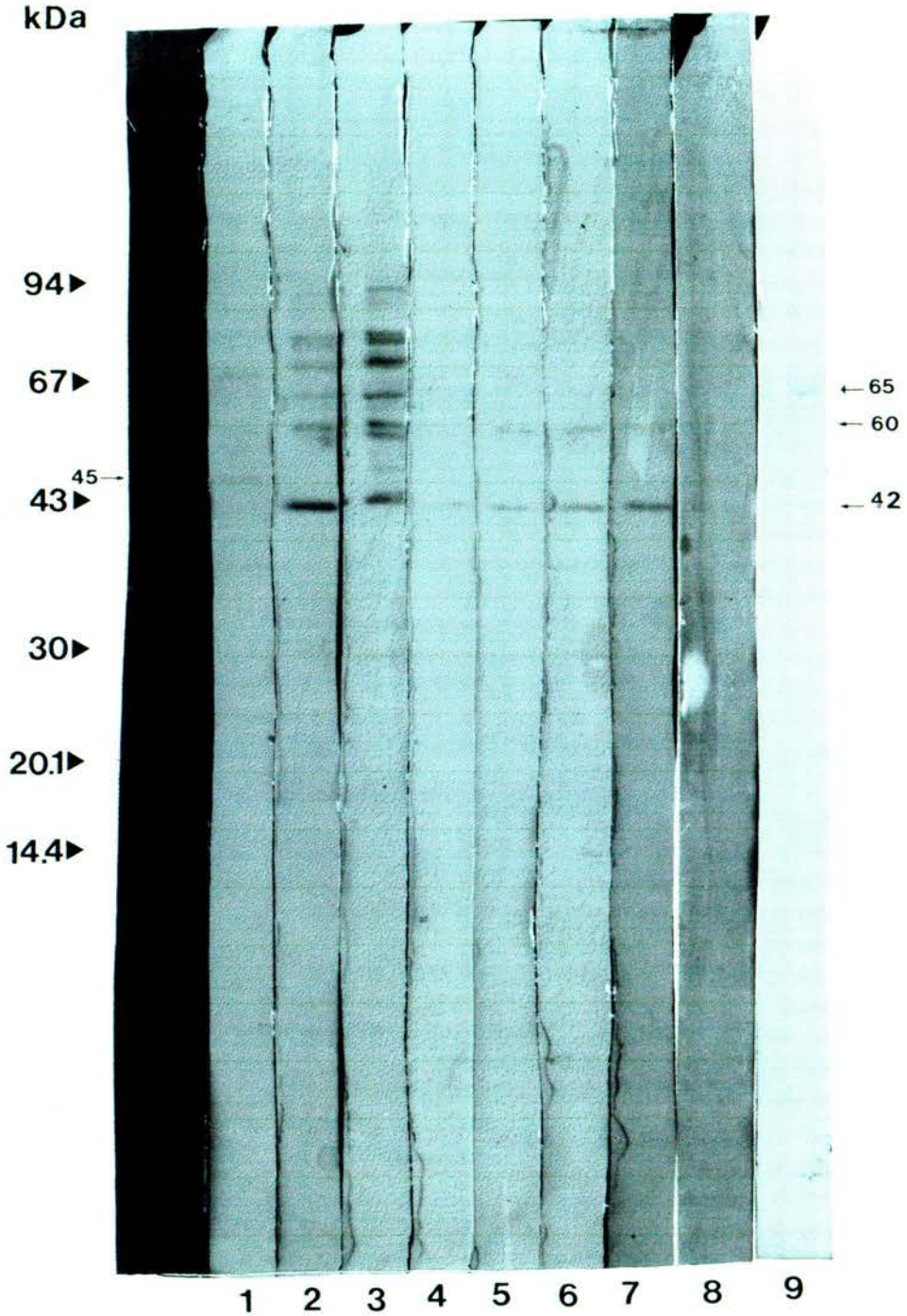


Fig 6.10 Western immuno-blotting of *B.bigemina* (Mexico stock) antigens probed with a pool of anti-*B.bigemina* sera (A), a pool of anti-*B.bovis* sera (B), and a pool of pre-infection sera(C)

Standard molecular markers are indicated on the left side.

Arrows on the right indicate faint bands recognised by pre-infection sera and anti-*B.bovis* sera.

Fig 6.10

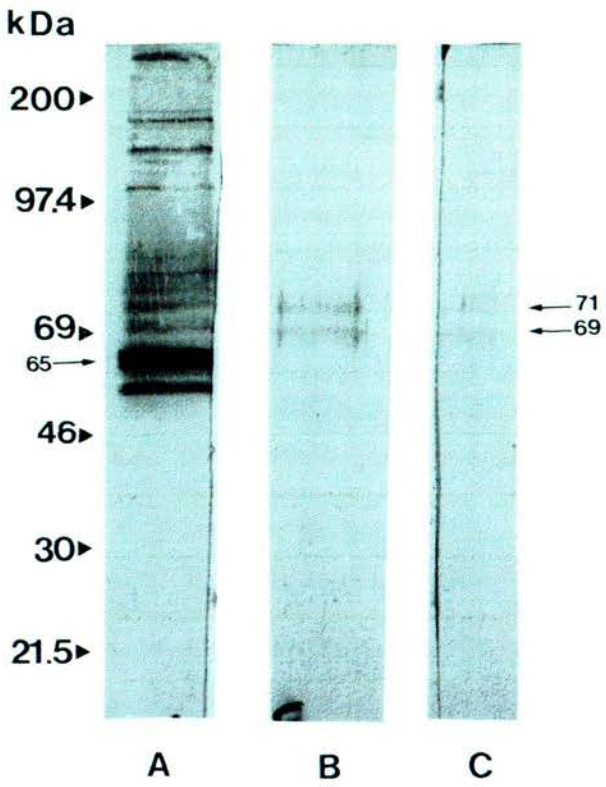


Table 6.3 Summary of protein bands of *B.bigemina* antigens detected by Western immuno-blotting.

Antigen molecular weight (kDa)	Sera					
	anti-*B.bigemina*		anti-*B.bovis*		pre-infection	
	Kenya	Mexico	Kenya	Mexico	Kenya	Mexico
255		▬				
230		—				
195		—				
185		—				
162		▬				
152		—				
148		—				
128		▬				
94	—					
88		—				
83		—				
80	—	—				
79	▬					
71	—	—		—		—
69		—		—		—
65	—	▬	----------			
60	—					
59	—					
50		▬				
45	—					
42	▬					

6.6 ANALYSIS OF CULTURE SUPERNATANT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

6.6.1 Introduction

Proteins may be purified on the basis of their molecular dimensions by filtration through porous gels in a column. The rate at which a molecule moves down a gel column depends upon the number of beads that are entered. Large proteins, above the exclusion limit of the gel, do not enter the beads and move with the elution solution, whereas small molecules enter the beads and must traverse this space as well as the volume around the beads. In addition to the molecular weight of the molecules, other factors, such as the molecular shape and degree of hydration, are important in governing the entry of molecules into the gel (Hudson & Hay, 1989). The HPLC system became operational in 1980 after the development of uniform rigid particles for use as chromatographic supports which enable high solvent flows to be maintained by the application of high pressure. A general system for HPLC consists of a pump that can deliver the eluant mixture at accurate flow rates of 0.1-1.0 ml/minute, an injection valve for application of samples, an optical flow-through cell compatible with an ultra-violet detector, a chart-recorder and the gel columns that cover the desired molecular weight range (Welling & Welling-Wester, 1989).

Separation of proteins may also be achieved by an ion exchange chromatography procedure, in which proteins are bound electrostatically onto an ion exchange matrix bearing an opposite charge. The degree of binding depends upon the protein charge density. Proteins are then eluted differentially by either increasing the ionic strength of the medium or altering the pH (Hudson & Hay, 1989). The high performance ion-exchange fractionation is based on these same principles, but using a packed column of particulate material consisting of a rigid, polymeric substrate, to which charged groups are bound (fixed charges). Proteins and peptides (mobile ions) will attach to the fixed charges via ionic interactions. This type of fractionation usually requires the use of a pH and/or ionic strength gradient to achieve the best resolution (Henry, 1989).

The experiments described in this section report results obtained from fractionation of culture supernatants of *B.bovis* and *B.bigemina* cultures with the aim of identifying relevant species-specific exoantigens which could be used for the development of specific ELISAs.

6.6.2 Materials and methods

6.6.2.1 Albumin removal and concentration of culture supernatants

Prior to collection of culture supernatants, *B.bovis* (Kwanyanga stock) and *B.bigemina* (Mexico stock) were cultured for 48 hours in M199-HS and M199-ICS, respectively (see table 3.2), which contained 5% NBS instead of the usual concentration of 40%. This was because, in a preliminary experiment, samples of culture supernatants containing 40% NBS showed disturbance of protein bands, when analysed by SDS-PAGE, primarily due to the high concentration of albumin.

An uninfected RBC suspension was cultured under the same conditions as *B.bovis* and *B.bigemina* to provide a control of non-infected culture supernatant. Culture suspensions were centrifuged at 1,600 x g for 10 minutes at 4° C, the supernatants collected and filtered through a 0.22 μm filter (Millipore). One volume of each filtered supernatant was then mixed with 5 volumes of Affigel blue (Bio-Rad) to remove serum albumin. The resulting suspensions were mixed overnight at room temperature on a blood cell suspension mixer. The suspensions were then processed by centrifugation as before and the resulting supernatants filtered as described above.

The efficacy of the Affigel blue in removing serum albumin was evaluated by SDS-PAGE and Coomassie blue staining of the samples before and after Affigel treatment.

After albumin removal, the supernatants were concentrated five fold by mixing approximately 5 ml of each supernatant with 0.92 g of Lyphogel (Gelman Instruments Company) over a period of 4 hours at room temperature. The concentrated supernatants were centrifuged (10,000 x g for 5 minutes), transferred to dialysis tubes (molecular cut-off 14,000 daltons) and dialysed overnight against size exclusion buffer (Appendix 1). Culture supernatant materials prepared in this way were used as starting material for HPLC fractionations.

6.6.2.2 Size exclusion fractionation

Concentrated culture supernatants from *B.bovis*, *B.bigemina* and uninfected RBC cultures were fractionated according to molecular size by HPLC using isocratic conditions (Welling & Welling-Wester, 1989). One ml of each sample was applied in column elution buffer (Appendix 1) using an injection loop. A 7.5 x 300 mm Biosep-SEC-S 300 column (Phenomenex) was used throughout the analysis. Samples were eluted from the column over a period of 30 minutes using elution buffer at a flow rate of 0.6 ml/min. The column eluate was

collected into 1.7 ml microcentrifuge tubes at 1 minute intervals during the chromatography. The absorbance of the column eluate was monitored continuously at 280 nm.

A sample of each fraction with absorbance greater than 0.05 was then run under reducing conditions in gradient (8 to 25%) SDS-PAGE using the Phast-gel system (Pharmacia). Gels were stained with silver nitrate according to the manufacture's instructions for detecting protein bands. Standard molecular weight markers (Bio-Rad, broad range) were included in each gel to enable estimation of molecular weights of bands.

Fractions containing protein bands detectable by silver staining were analysed by Western immuno-blotting against pools of either species-specific antisera or pre-infection sera. The fractions containing antigens were then further analysed by an ion exchange HPLC.

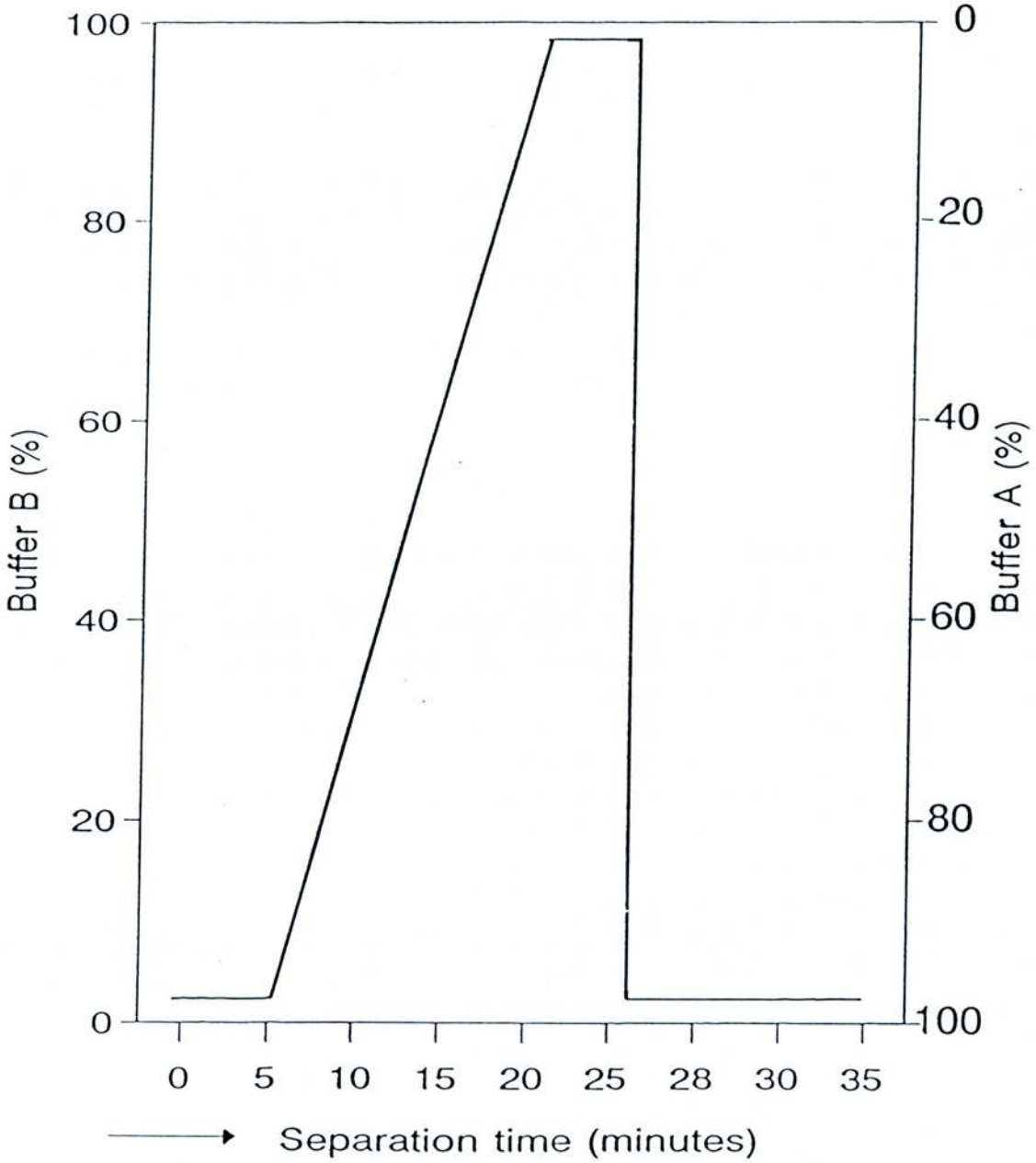
6.6.2.3 Ion exchange fractionation

Samples were subjected to ion-exchange by HPLC using a 75 x 7.5 mm Spherogel TSK DEAE-5 PW column (Beckman). One ml of each sample was applied to the column in buffer A (Appendix 1) using an injection loop. Proteins were eluted from the column using a continuous gradient generated using two buffers of pH 7.0 and 8.3 (buffer A and buffer B, Appendix 1) at a flow rate of 1 ml/min over a period of 30 minutes, as shown in Fig 6.11. The column eluate was collected into 1.7 ml microcentrifuge tubes at 1 minute intervals during the chromatography. The absorbance of the column eluate was monitored continuously at 280 nm. Fractions eluted from the column with absorbance greater than 0.05 were then analysed by SDS-PAGE, and Western immuno-blotting as previously described.

Samples of purified bovine IgG (Sigma) and NBS were separated by SDS-PAGE on the same gel as selected fractions of *B.bovis* culture supernatant obtained after HPLC, blotted and reacted with either each pool of sera (pre-infection, anti-*B.bovis*, *B.bigemina*) or with PBS in order to detect reactions from the anti-bovine IgG conjugate against IgG and other serum components present in fractions of culture supernatants.

Fig 6.11 Continuous gradient used in ion-exchange fractionations of culture supernatants

Fig 6.11



From time 0 to 5 minutes the column was eluted with 100% buffer A.
From time 5 to 20 minutes the column was eluted with a linear gradient of 0 to 100% buffer B.
From time 20 to 25 minutes the column was eluted with 100% buffer B.
From time 25 to 26 minutes the column was eluted with a linear gradient of 0 to 100% buffer A.
From time 26 to 30 minutes the column was eluted with 100% buffer A.

6.6.3 Results

6.6.3.1 Albumin removal and concentration of culture supernatant

The treatment of culture supernatant with Affigel Blue resulted in an approximately 10 fold reduction in concentration of the band corresponding to bovine serum albumin (66 kDa), as shown by Coomassie blue staining of samples before and after treatment (Fig 6.12, lanes 1 and 2; 3 and 4 respectively).

Treatment of culture supernatant with Lyphogel resulted in concentration of proteins in the samples as shown by more intensive Coomassie blue staining of bands after concentration (Fig 6.12, lanes 5 and 6).

After albumin removal some protein bands in a range between 200-90 and 55-29 kDa were absent. Subsequent concentration with lyphogel recovered most of the bands in the 200-90 kDa range but only some of the bands in the lower molecular range of 55-29 kDa. No detectable differences were seen between the total protein profiles of uninfected and infected culture supernatants.

Fig 6.12 Coomassie blue staining of a 10% homogeneous gel showing culture supernatants before and after treatment with Affigel and Lyphogel.

Lanes 1 - supernatant from an uninfected culture containing 5% serum.

2 - supernatant from a *B.bovis* culture containing 5% serum.

3 - uninfected culture supernatant after treatment with Affigel.

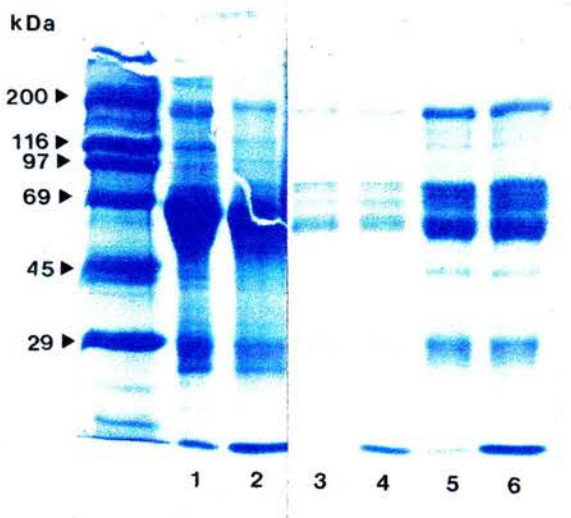
4 - *B.bovis* culture supernatant after treatment with Affigel.

5 - uninfected culture supernatant after treatment with Affigel and concentration with Lyphogel.

6 - *B.bovis* culture supernatant after treatment with Affigel and concentration with Lyphogel.

Standard molecular weight markers are indicated on the left.

Fig 6.12



6.6.2.2 Fractionation of *B.bovis* culture supernatant

Two peaks of absorbance were obtained from the fractionation of the uninfected culture supernatant after the size exclusion procedure (Fig 6.13). The first peak (A) was detected in fractions 9 to 13 and the second (B) in fractions 13 to 19. Fractionation of *B.bovis* culture supernatant gave three peaks. The first peak (A) was detected in fractions 9 to 13, coinciding with the uninfected supernatant; the second peak (B) was detected in fractions 13 to 17 and was not as distinct as the one of uninfected supernatant. A third peak (C) was detected in fractions 16 to 19 of the *B.bovis* supernatant and was not clearly resolved.

SDS-PAGE analysis of *B.bovis* supernatant showed that the starting material used for HPLC fractionation contained at least 17 protein bands with molecular weights ranging from 200 to 6.5 kDa (Fig 6.14 A, lane 11). Fractions 21 and 20 contained protein bands of molecular weights greater than 90 kDa (Fig 6.14 A, lanes 1 and 2); fractions 19-14 contained proteins in a range between 200 and 20 kDa (Fig 6.14 A, lanes 3-8); and fractions 13 and 12 both contained a band of molecular weight 60 kDa (Fig 6.14, lanes 9 and 10).

The overall SDS-PAGE pattern from the uninfected supernatant was similar to that from the *B.bovis* supernatant, with minor differences. Fractions 19 and 18 contained fewer bands in the low molecular weight range, and fewer bands in the high molecular weight region (Fig 6.14 B, lanes 3 and 4).

Western immuno-blotting analysis of nine fractions (11, 14, 15-21, as indicated in Fig 6.14 A) of *B.bovis* supernatant against the pool of anti-*B.bovis* sera showed three antigenic bands at 120, 90 and 55 kDa in fractions 15 to 18 (Fig 6.15 A). The first immuno-blotting analysis of these fractions showed that the 55 kDa protein in fractions 16 and 17 was reactive with the pool of anti-*B.bovis* sera but not reactive with the pool of pre-infection sera. For this reason fractions 16 and 17 were selected for further fractionation by ion exchange. However these results were not reproducible on other occasions and the same band (55 kDa) was reactive in all fractions when probed with the pool of pre-infection sera, as shown in Fig 6.15 B.

Fig 6.13 Fractionation of *B.bovis* culture supernatant by HPLC (size exclusion).

Fig 6.13

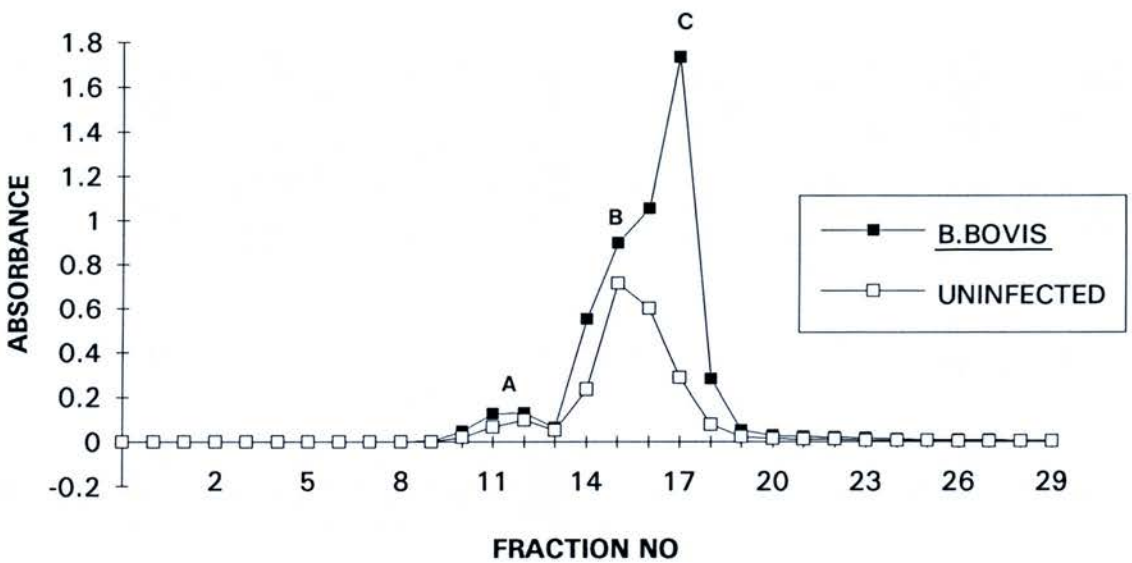

C
B
A
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
ABSORBANCE
2
5
8
11
14
17
20
23
26
29
FRACTION NO
B.BOVIS
UNINFECTED

Fig 6.14 Silver staining of a 4-20% gradient gel containing fractions of supernatant of a *B.bovis* culture (A) and an uninfected culture (B) obtained after HPLC fractionation by size exclusion.

Lanes:
1 - fraction 21
2 - fraction 20
3 - fraction 19
4 - fraction 18
5 - fraction 17
6 - fraction 16
7 - fraction 15
8 - fraction 14
9 - fraction 13
10- fraction 12
11- starting culture supernatant
12- standard molecular weight markers

Fig 6.14

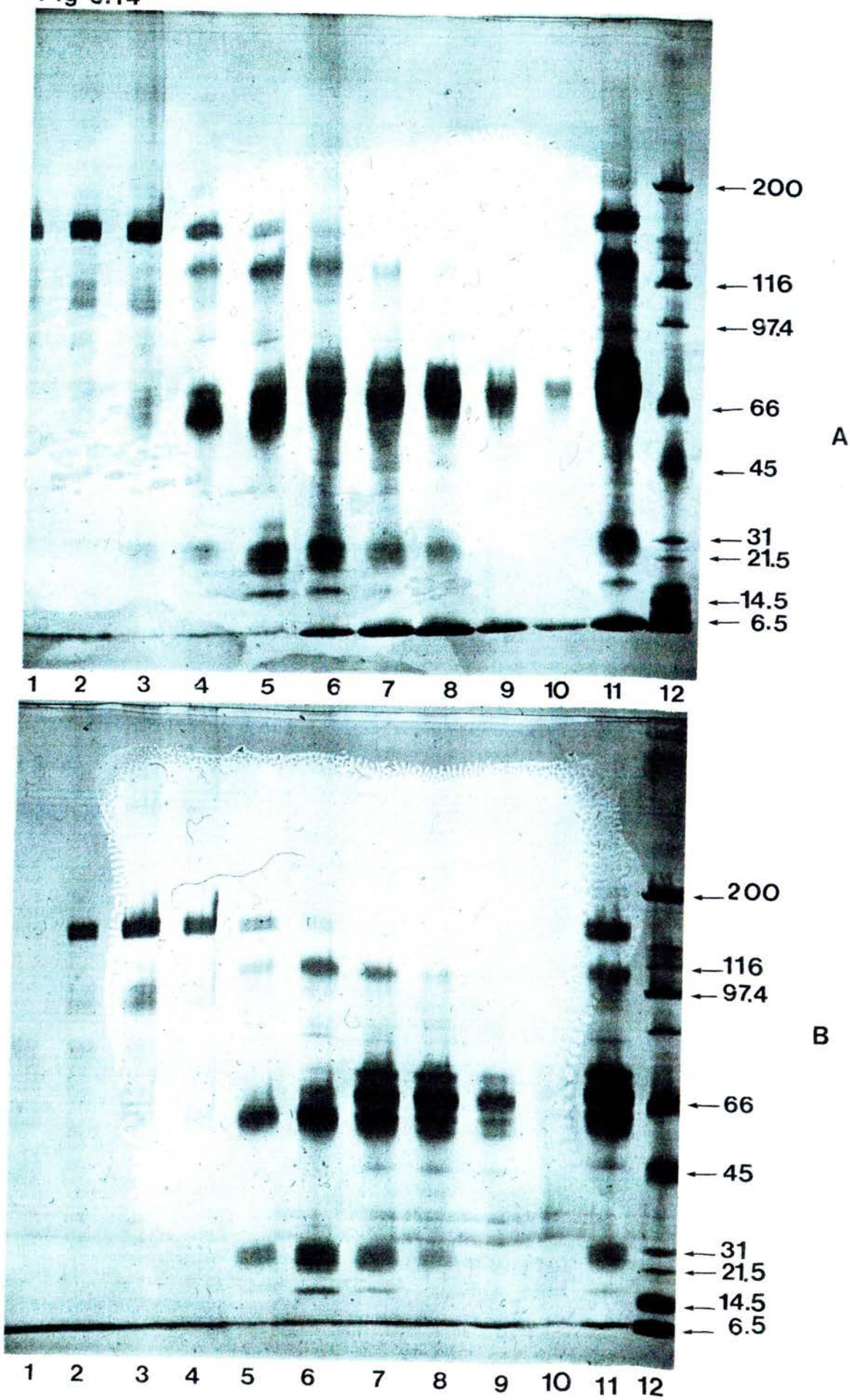

200
116
97.4
66
45
31
21.5
14.5
6.5
A
1 2 3 4 5 6 7 8 9 10 11 12
200
116
97.4
66
45
31
21.5
14.5
6.5
B
1 2 3 4 5 6 7 8 9 10 11 12

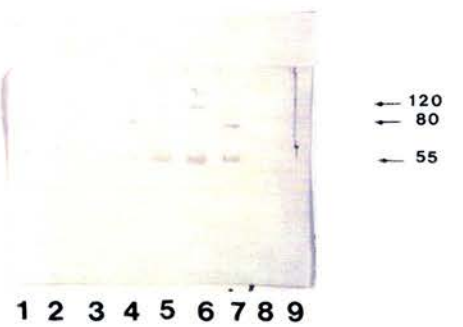
Fig 6.15 Western immuno-blotting of fractions of *B.bovis* culture supernatant against either a pool of anti-*B.bovis* sera (A) or a pool of pre-infection sera (B).

Lanes:
1 - fraction 21
2 - fraction 20
3 - fraction 19
4 - fraction 18
5 - fraction 17
6 - fraction 16
7 - fraction 15
8 - fraction 14
9 - fraction 11

Standard molecular weight markers are indicated on the left.

Fig 6.15

A



B

The ion exchange analysis of *B.bovis* fractions 16 and 17 resulted in 30 sub-fractions from each fraction. The corresponding fractions 16 and 17 from the uninfected supernatant were also analysed by ion exchange.

Ion exchange analysis of *B.bovis* supernatant fraction 16 and the corresponding fraction of the uninfected supernatant gave similar chromatography traces, as shown in Fig 6.16. Two peaks of absorbance were detected for both the *B.bovis* and the uninfected fraction 16. The first peak was detected in sub-fractions 13 to 18, and the second in sub-fractions 19 to 24.

Eleven of the *B.bovis* supernatant sub-fractions (4, 5, 15-17, 21-24, 27 and 28) were analysed by SDS-PAGE and protein bands were detected in fractions 15 to 28, as shown in Fig 6.17 (arrows on the left). Sub-fractions 22 to 28 were then selected for further analysis by Western immuno-blotting against the pools of both pre infection and anti-*B.bovis* sera.

Results from this analysis showed that both pre and post-infection pools reacted with protein bands at molecular weights 120 and 55 kDa in both sub-fractions 23 and 22 (Fig 6.18 A and B, lanes 6 and 7), and the 55 kDa protein in sub-fractions 24 to 28 (Fig 6.18 A and B, lanes 1-5).

The second fraction selected for ion exchange sub-fractionation (fraction 17) also showed a pattern of absorbance of sub-fractions similar to that of the corresponding fraction from uninfected supernatant (Fig 6.19). In both samples peaks of absorbance were detected in sub-fractions 12 to 29. However, they were not clearly resolved.

Silver staining detected protein bands in sub-fractions 18 to 26, as shown in Fig 6.20 (arrows). Sub-fractions 20 to 26 were then selected for further analysis by Western immuno-blotting.

This analysis also resulted in detection of a reactive protein band (at 55 kDa) in all sub-fractions, except in sub-fraction 20 (Fig 6.21 A). However the same band was also recognised by the pool of pre-infection sera (Fig 6.21 B).

Fig 6.16 Sub-fractionation by HPLC (ion exchange) of fraction 16 of *B.bovis* culture supernatant.

Fig 6.16

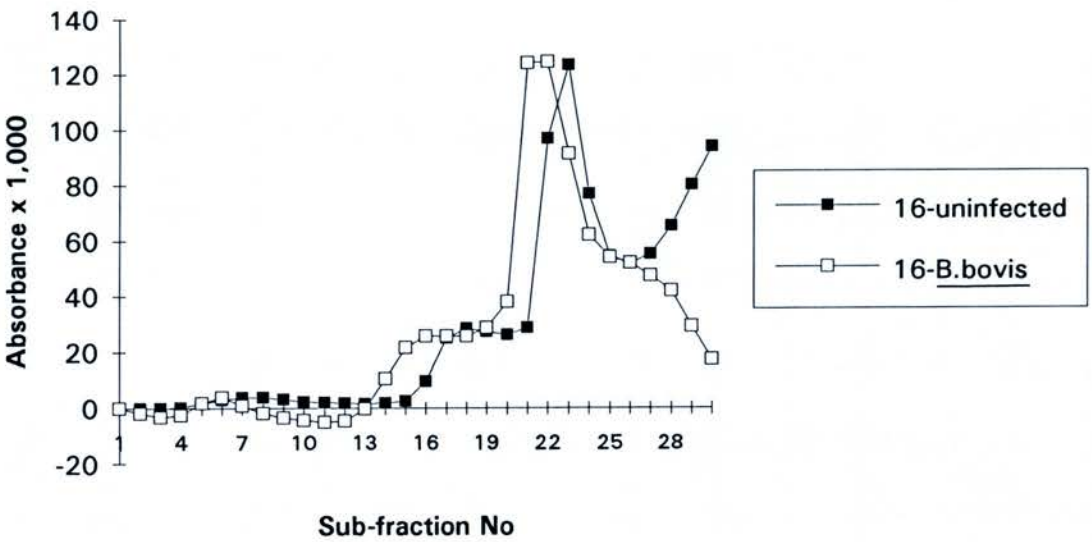

140
120
100
80
60
40
20
0
-20
Absorbance x 1,000
1
4
7
10
13
16
19
22
25
28
Sub-fraction No
16-uninfected
16-B.bovis

Fig 6.17 Silver staining of a 4-20% gradient gel containing sub-fractions of fraction number 16 of *B.bovis* culture supernatant obtained after HPLC fractionation (ion exchange).

Lanes:
1 - sub-fraction 28
2 - sub-fraction 27
3 - sub-fraction 24
4 - sub-fraction 23
5 - sub-fraction 22
6 - sub-fraction 21
7 - sub-fraction 17
8 - sub-fraction 16
9 - sub-fraction 15
10- sub-fraction 5
11- sub-fraction 4
12- standard molecular weight markers

Fig 6.17

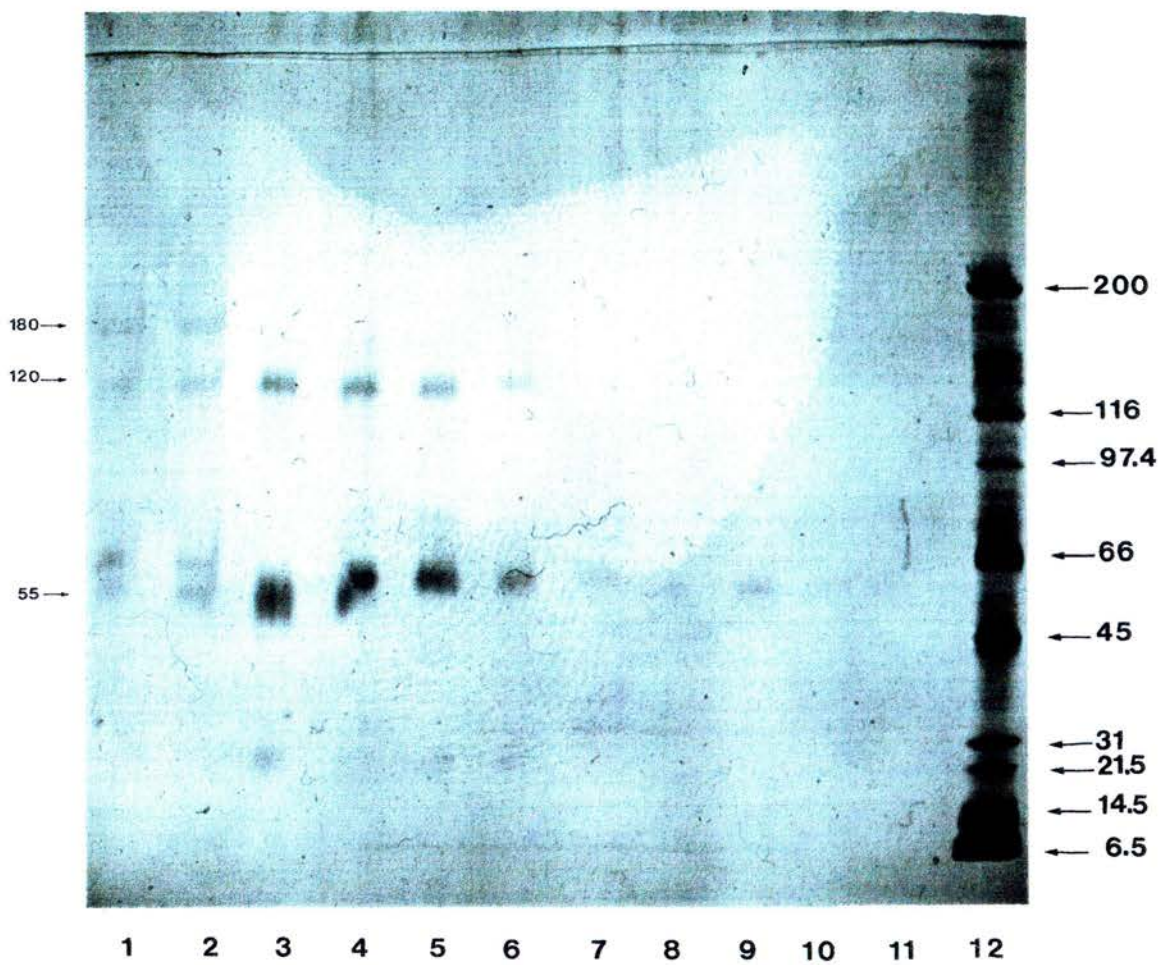
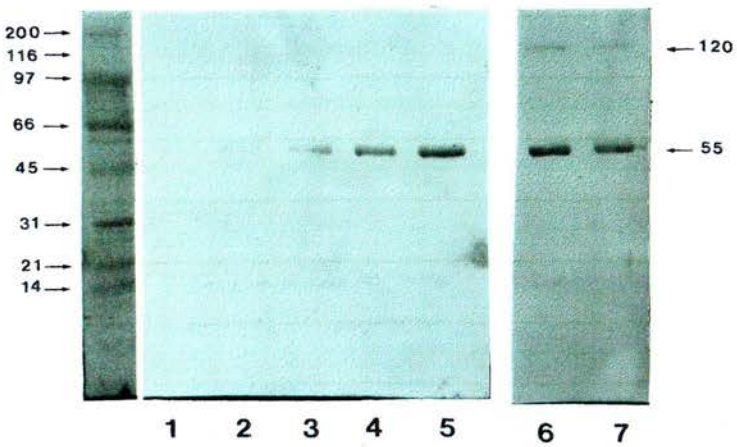


Fig 6.18 Western immuno-blotting of sub-fractions of fraction number 16 of *B.bovis* culture supernatant against either a pool of anti-*B.bovis* sera (A) or a pool of pre-infection sera (B).

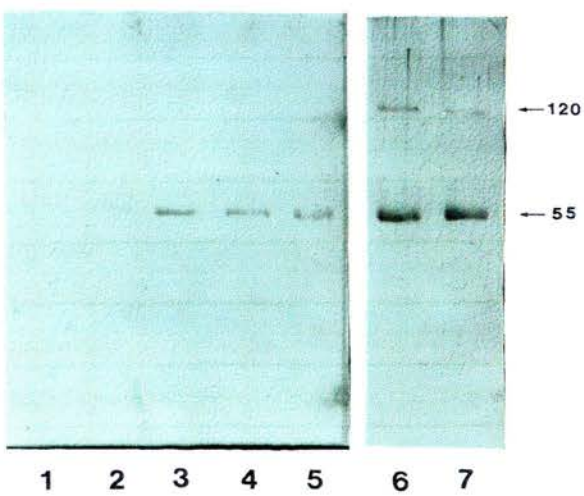
Lanes:
1 - sub-fraction 28
2 - sub-fraction 27
3 - sub-fraction 26
4 - sub-fraction 25
5 - sub-fraction 24
6 - sub-fraction 23
7 - sub-fraction 22

Standard molecular weight markers are indicated on the left.

Fig 6.18



A



B

Fig 6.19 Sub-fractionation by HPLC (ion exchange) of fraction 17 of *B.bovis* culture supernatant.

Fig 6.19

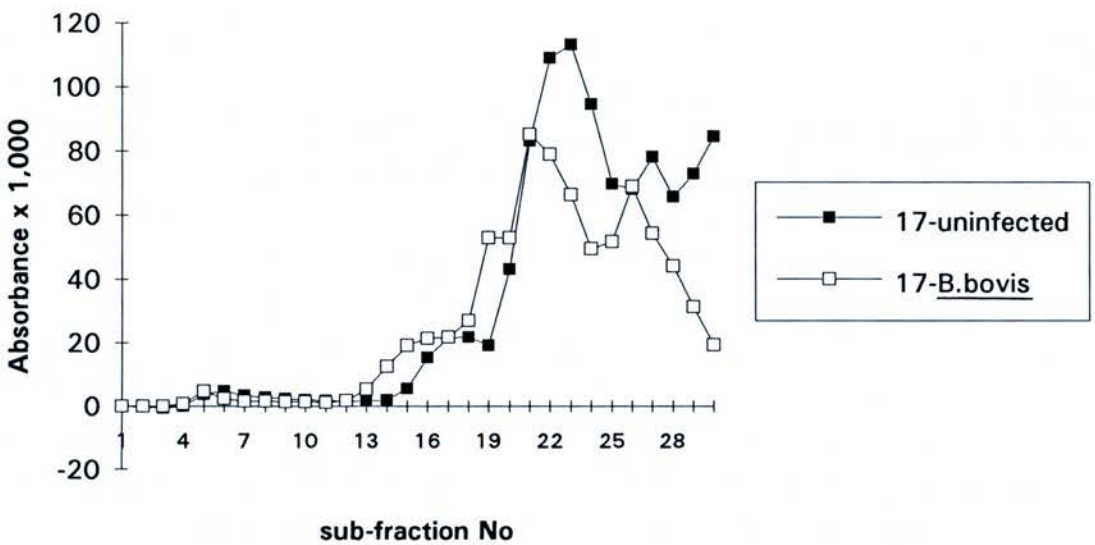

120
100
80
60
40
20
0
-20
Absorbance x 1,000
1
4
7
10
13
16
19
22
25
28
sub-fraction No
17-uninfected
17-B.bovis

Fig 6.20 Silver staining of a 4-20% gradient gel containing sub-fractions of fraction number 17 of *B.bovis* culture supernatant obtained after HPLC fractionation (ion exchange).

Lanes:
1 - sub-fraction 26
2 - sub-fraction 25
3 - sub-fraction 22
4 - sub-fraction 21
5 - sub-fraction 20
6 - sub-fraction 18
7 - sub-fraction 17
8 - sub-fraction 16
9 - sub-fraction 6
10-sub-fraction 5
11-sub-fraction 4
12-standard molecular weight markers

Fig 6.20

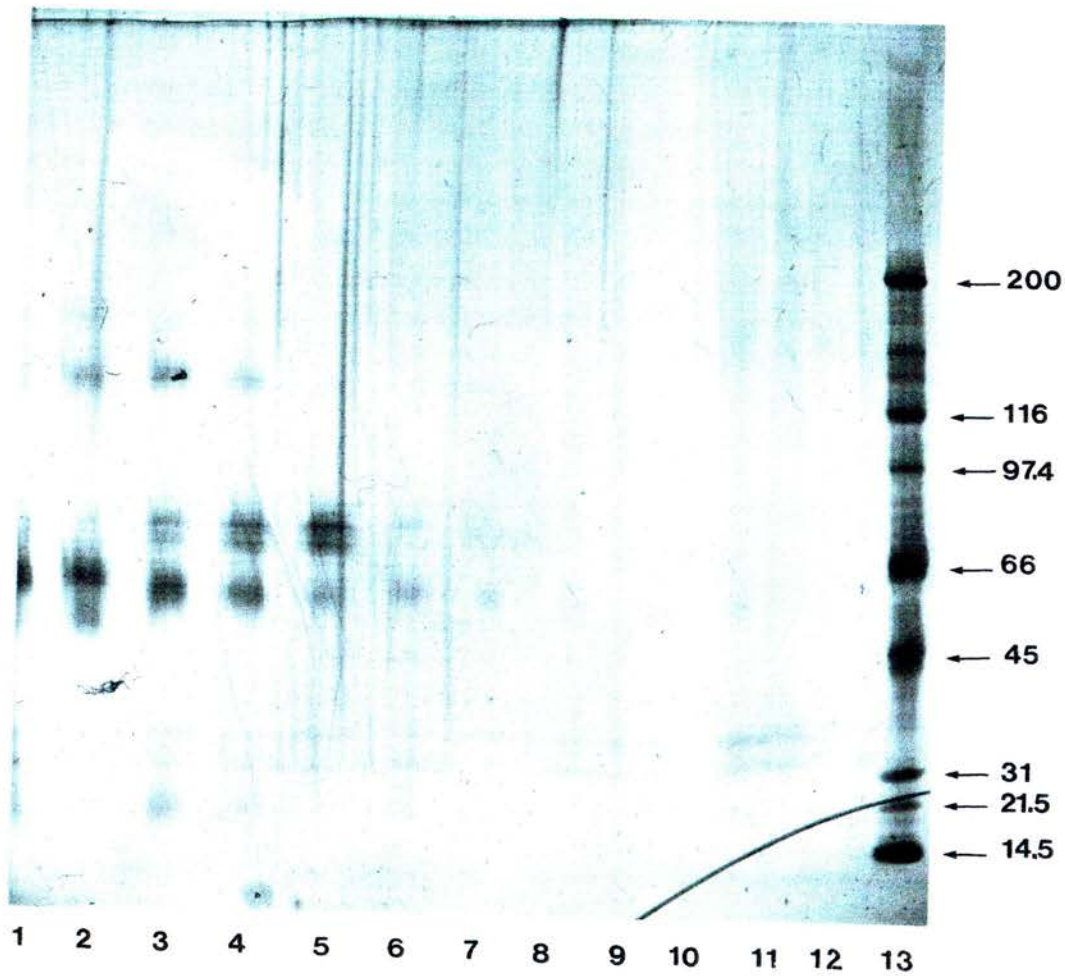
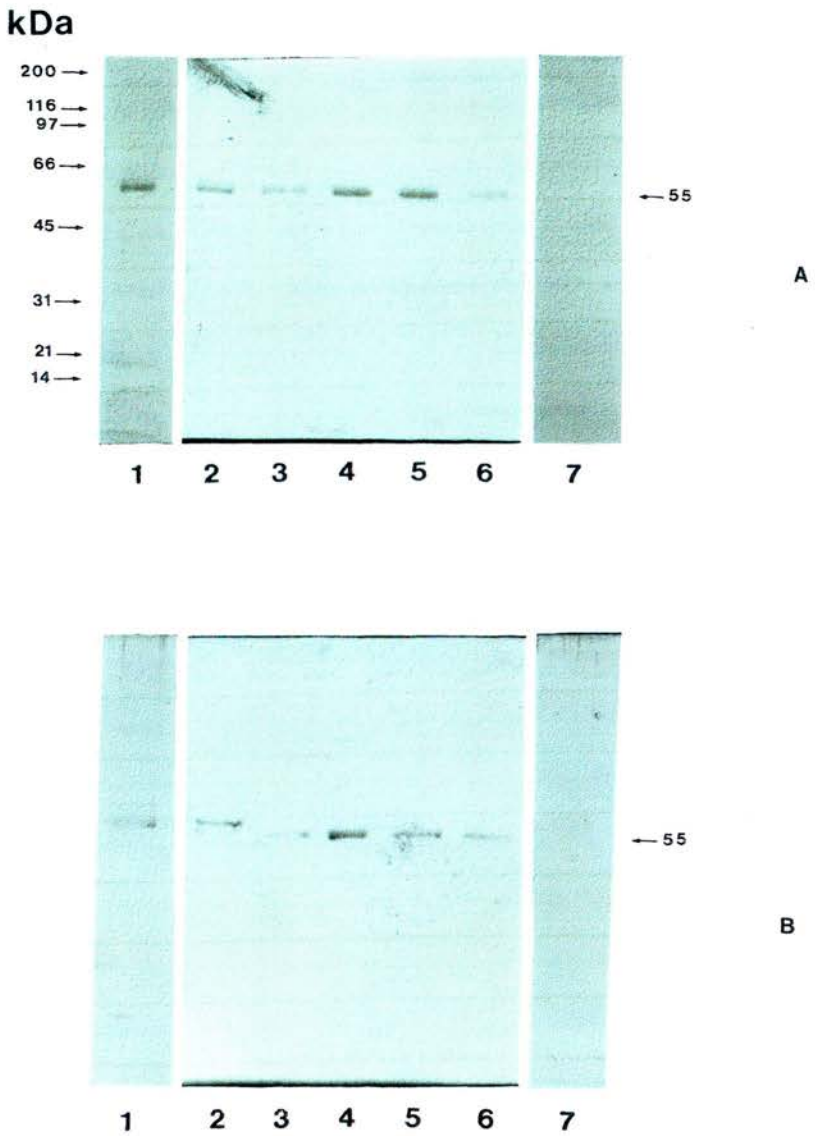


Fig 6.21 Western immuno-blotting of sub-fractions from fraction number 17 of *B.bovis* culture supernatant against either a pool of anti-*B.bovis* sera (A) or a pool of pre-infection sera (B).

Lanes:
1 - sub-fraction 26
2 - sub-fraction 25
3 - sub-fraction 24
4 - sub-fraction 23
5 - sub-fraction 22
6 - sub-fraction 21
7 - sub-fraction 20

Standard molecular weight markers are indicated on the left.

Fig 6.21



6.6.3.3 Fractionation of *B.bigemina* culture supernatant

Three peaks of absorbance were detected after size exclusion fractionation of *B.bigemina* supernatant (Fig 6.22). The first peak (A) was detected in fractions 12 to 16, the second (B) in fractions 16 to 23 and the third (C) in fractions 27 to 29.

Silver staining of acrylamide gels detected protein bands in fractions 14 to 26 (Fig 6.23 A and B).

When fractions 14, 15 and 18-24 were further analysed by Western immuno-blotting against the pool of anti-*B.bigemina* sera, two reactive bands (at molecular weights 120 and 55 kDa) were detected (Fig 6.24 A). However, the same bands were also detected by the pool of pre-infection sera (Fig 6.24 B).

Western immuno-blotting analysis of NBS and purified IgG resulted in detection of two bands of molecular weights corresponding to those detected in fractions of *Babesia* culture supernatants suggesting that these were due to reaction of the anti-bovine IgG conjugate against bovine IgG (Fig 6.25). With these results, no further fractionations were carried out with *B.bigemina* culture supernatant.

Fig 6.22 Fractionation of *B.bigemina* culture supernatant by HPLC (size exclusion).

Fig 6.22

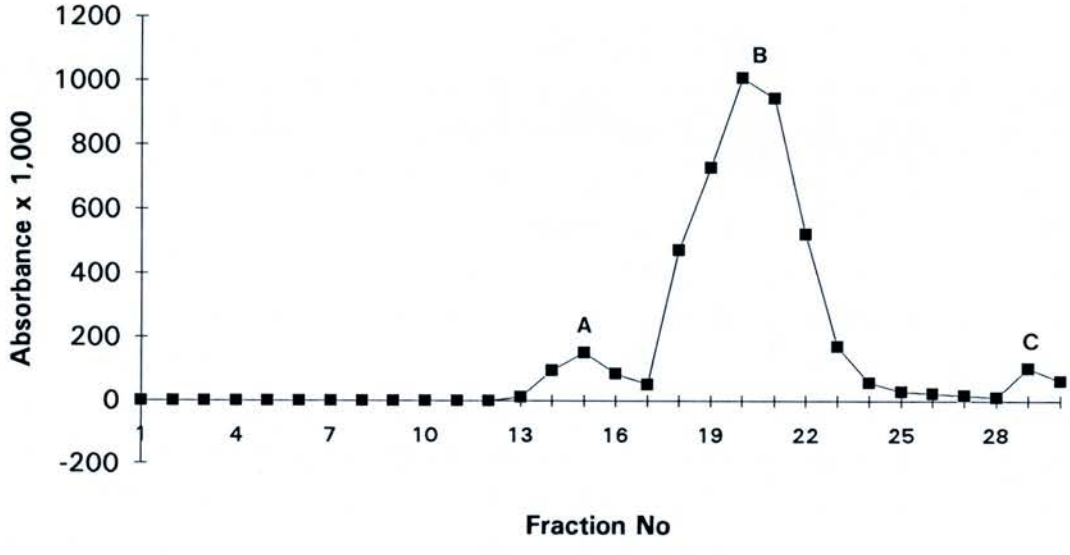

1200
1000
800
600
400
200
0
-200
Absorbance x 1,000
A
B
C
1
4
7
10
13
16
19
22
25
28
Fraction No

Fig 6.23 Silver staining of a 4-15% gradient gel containing fractions of *B.bigemina* culture supernatant obtained after HPLC fractionation by size exclusion.

Lanes:
1 - fraction 11
2 - fraction 12
3 - fraction 13
4 - fraction 14
5 - fraction 15
6 - fraction 16
7 - fraction 17
8 - fraction 18
9 - fraction 19
10-fraction 20
11-fraction 21
12-fraction 22
13-fraction 23
14-fraction 24
15-fraction 25
16-fraction 26
17-fraction 27
18-fraction 28
19-fraction 29
20-fraction 30

Fig 6.23

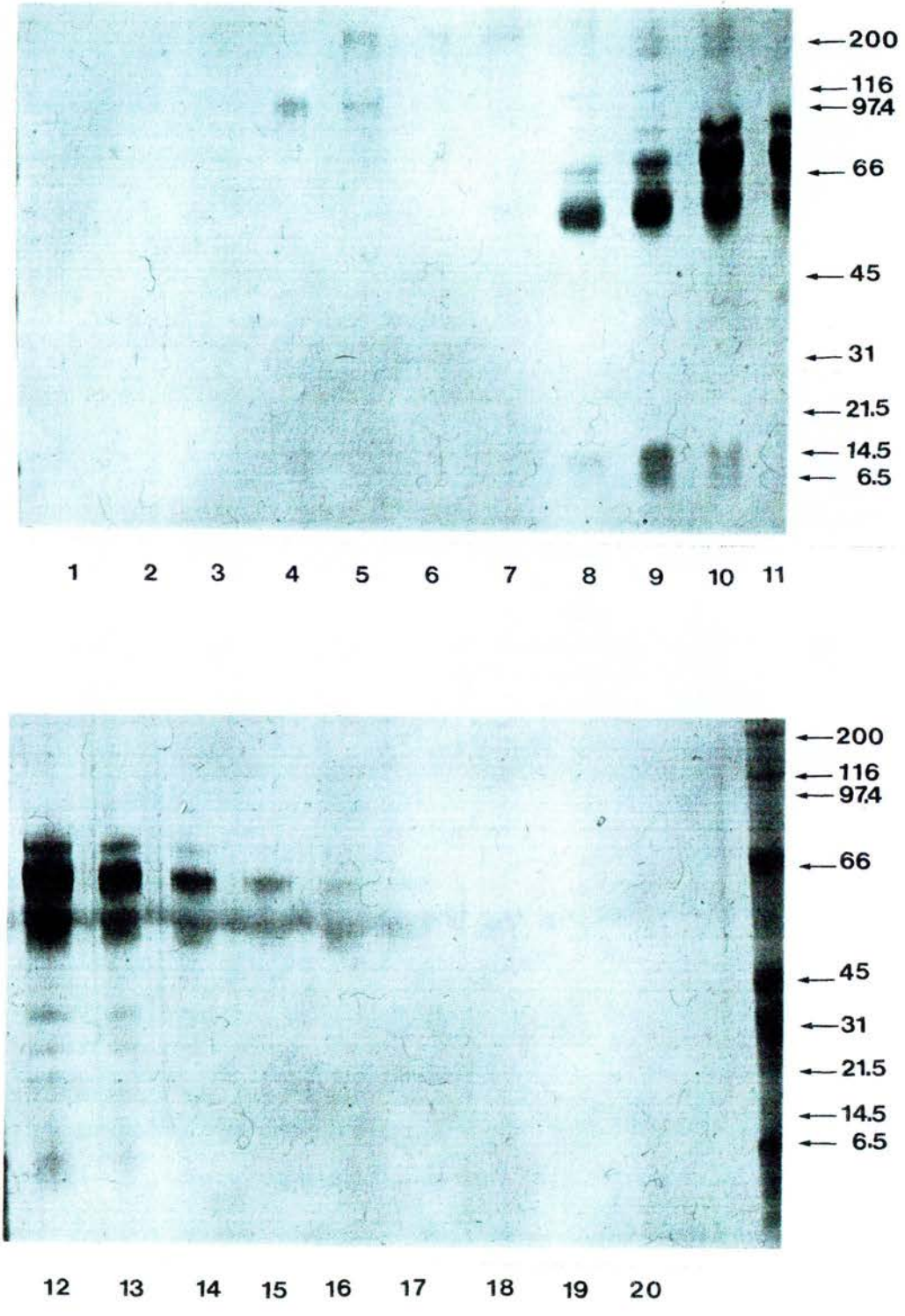

200
116
97.4
66
45
31
21.5
14.5
6.5
1 2 3 4 5 6 7 8 9 10 11
200
116
97.4
66
45
31
21.5
14.5
6.5
12 13 14 15 16 17 18 19 20

Fig 6.24 Western immuno-blotting of fractions of *B.bigemina* culture supernatant against either a pool of anti-*B.bigemina* sera (A) or a pool of pre-infection sera (B).

Lanes:
1 - fraction 24
2 - fraction 23
3 - fraction 22
4 - fraction 21
5 - fraction 20
6 - fraction 19
7 - fraction 18
8 - fraction 15
9 - fraction 14

Fig 6.24

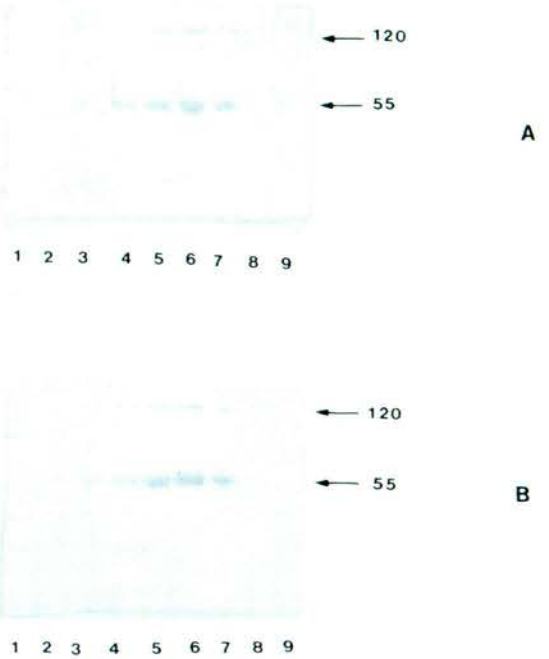
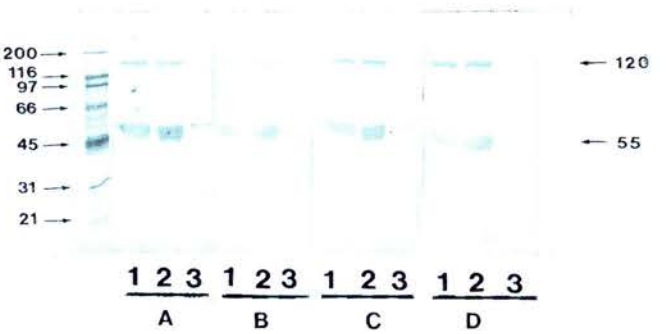


Fig 6.25 Western immuno-blotting of NBS, purified bovine IgG and fraction 17 of *B.bovis* supernatant against a pool of anti-*B.bovis* sera (A), a pool of anti-*B.bigemina* sera (B), a pool of pre-infection sera (C) and PBS control (D).

Lanes:
1 - NBS
2 - bovine IgG
3 - fraction 17 of *B.bovis* supernatant

Molecular weight markers are indicated on the left.

Fig 6.25



6.7 DISCUSSION AND CONCLUSIONS

In the studies described here immunodominant antigens of both *B.bovis* and *B.bigemina* parasites were identified by immuno-blotting using immune calf sera produced by experimental inoculation of iRBC, and sera from cattle which had undergone natural infections under field conditions.

The study on antigen preparation showed that, for both parasites, the strongest reactions were obtained in blots containing samples which had been prepared using either pelleted parasites or iRBC to which no enzyme inhibitors had been added and which had not been lysed before freezing (Fig 6.1). In such antigen preparations solubilisation of proteins was achieved with the addition of reducing sample buffer, which contained a detergent (SDS) and a reducing agent (mercaptoethanol).

Although samples without enzyme inhibitors were more concentrated than those to which enzyme inhibitors were added, and therefore the stronger reactions observed with them might simply have been due to their higher protein concentrations, it appears that there was no need to include enzyme inhibitors in the preparation of *Babesia* samples for electrophoresis. Addition of enzyme inhibitors would be of more importance if samples were to be subjected to sequential freezing/thawing procedures which might result in degradation of proteins by parasite proteinases. Under the conditions used here, there was little time for any protein degradation since the parasite preparations were kept frozen until just before use and once they were thawed, the SDS reducing sample buffer was added immediately.

The results from this study showed that solubilisation and separation of *B.bovis* and *B.bigemina* proteins by SDS-PAGE was satisfactory when SDS-reducing sample buffer was added directly to frozen concentrated parasites or iRBC without any previous lysis, and that the proteins separated by this method were recognised by immune sera. This method was therefore used for the preparation of *Babesia* antigens for immuno-blotting studies.

Polyclonal sera contain a mixture of different populations of antibodies that are specific for different antigens. When such antisera are used in immunochemical characterisation of antigens interpretation of results can be difficult. The development of techniques for hybridoma production has allowed the preparation of McAbs which are homogeneous, have a defined specificity and can theoretically be produced in unlimited quantities (Harlow & Lane, 1988).

It was expected that, in the present study, a panel of McAbs would be useful in identifying *B.bovis*-specific components. Once relevant epitopes were identified, it was hoped that they could be purified from crude parasite extracts by the use of the corresponding McAb in an affinity purification procedure.

The IFAT analysis of the panel of McAbs showed that all McAbs, with only one exception (McAb 2A11), reacted against all three stocks of *B.bovis*. This was encouraging since antigens conserved between isolates are necessary for diagnostic assays. Distinct patterns of staining were observed with each of the McAbs, which allowed their classification into three groups according to their specific reactivity against either parasite surface, parasite internal components, or parasite and iRBC components (Table 6.1).

Despite their reactivity in IFAT, only four McAbs (4F3:1, 1B8:1, 2A11 and 118.672) reacted in ELISA, with relatively low end point titres. All four ELISA reactive McAbs had reacted against either parasite internal components of iRBC or cytoplasm/membrane in IFAT. None of the McAbs that had reacted against parasite surface in IFAT reacted in ELISA. The differences in reactivity between IFAT and ELISA might be due to substantial differences between these two tests particularly in relation to the type of antigens used in each case. The IFAT uses as antigen the whole iRBC after fixation and therefore reactions detected may include ones directed against conformational components. In contrast, the ELISA uses as antigen a soluble parasite preparation which is usually obtained by sonication and therefore the original structure of parasite/RBC components is disrupted. This might be an explanation for the lack of reactivity in ELISA of McAbs which reacted against the parasite surface in IFAT. The reactivity of the McAbs in IFAT associated with the lack of reactivity of most of them in ELISA gives support to the hypothesis that these McAbs may react specifically against epitopes of conformational components (Harlow & Lane, 1988).

Further evidence for the lack of specific reactivity of these McAbs was observed in Western immuno-blotting, which also uses soluble parasite extracts as antigen. In the antigen preparations for immuno-blotting, the proteins are further reduced by mercaptoethanol present in the sample buffer and disrupted by boiling for 5 minutes. For the immuno-blotting analysis, the McAbs were pooled into groups of two or three. This approach was used based on findings that a combination of multiple McAbs allows formation of stable multivalent complexes, increasing the avidity for the antigen in question (Harlow & Lane, 1988).

The immuno-blotting analysis of four pools of McAbs showed a reaction against two protein bands (molecular weights of 195-200 kDa) (Fig 6.2), which did not appear to be parasite-specific components, as evidenced by their detection on several occasions by pools of pre-infection sera (see Fig 6.6 C), although the reaction from pre-infection sera was much fainter than those from the pools of McAbs. On the other hand, the anti-*T.evansi* McAb did not detect them. Amongst the four pools of McAbs, one reacted with a band with molecular weight of 58 kDa which was detected only in the Kwanyanga stock. Due to the fact that the McAbs had been raised against this stock of *B.bovis*, these results suggest that the 58 kDa band might be a Kwanyanga-specific epitope.

The immuno-blotting analysis using the panel of McAbs did not identify any stock-common antigenic determinant, thus none of the McAbs could be used to purify parasite-specific antigenic determinants using a McAb affinity purification procedure. Nevertheless, the McAbs which had given the strongest (brightest) reactions by IFAT (McAbs 3D42, 1C12:2, 3D6:1 and 4D1:3) and the ones which had resulted in highest ELISA titres (4F3:1 and 1B8:1) were selected for further investigation by immunoprecipitation of ^{35}S-labelled proteins as described in Chapter 7.

The analysis of the total protein profiles of preparations of the different stocks of *B.bovis* showed a large number of protein bands in Coomassie blue stained gels (Fig 6.3). The protein profiles of the stocks were very similar and no differences were detected between the parasite and the uninfected RBC preparations. These results could be due to presence of RBC contamination in the parasite preparations and for this reason, an uninfected RBC lysate was included as a negative control in immuno-blotting analysis. However, similarities between the profiles of infected and uninfected preparations may merely reflect a limitation of one-dimensional gel electrophoresis, which only separates the proteins according to their molecular weight. It was therefore not possible to determine if the bands with similar molecular weights in the uninfected and parasite preparations comprised the same proteins.

The Western immuno-blotting analysis of the *B.bovis* and uninfected RBC antigens, using calf sera produced by experimental inoculation of iRBC, resulted in identification of the major *B.bovis* immunodominant proteins and indicated that antigenic differences exist within the stocks used (Fig 6.4, 6.5 and 6.6). The Kwanyanga and the Mexico stocks consistently showed similar antigenic profiles throughout the study, whereas the Lismore stock was different from the other two stocks in relation to some antigens. Evidence for this was the differences found in antigen profiles when different calf sera were used to probe the three stock preparations on the same gel (Fig 6.4). Three antigens (molecular weights of 70, 39 and 37 kDa) present in the Mexico and Kwanyanga stocks were absent in the Lismore stock (Fig 6.4 A) and two antigens (molecular weights of 78 and 30 kDa) were present only in the Lismore stock (Fig 6.4 B). These findings give support to the belief that there is antigenic diversity amongst the *B.bovis* stocks (Kahl et al, 1983; Gill et al, 1987a; Timms et al, 1990). However, one should bear in mind, when interpreting the results of immuno-blot analysis, that differences observed in the antigen profiles between the stocks could in part be due to differences in the immune responses of individual calves against particular parasite components. It was not possible to explore this further with the limited number of stock-specific calf sera available for this study.

Further evidence of antigenic diversity amongst the stocks of *B.bovis* was seen when a pool of field sera from Brazil was used to probe the three antigens. Results from this analysis again

showed antigenic differences between the stocks, with two antigens (39-37 kDa) not being detected in the Lismore stock while the 30 kDa antigen was only detected in Lismore. This analysis also indicated that the Brazilian field sera reacted more strongly with the Kwanyanga antigen (Fig 6.6, lane 1 A) than with the Mexico and Lismore antigens (Fig 6.6, lanes 2 and 3 A). This suggests that the Brazilian field stocks of *B.bovis* might be more antigenically similar to this stock than to the other two stocks.

Antigenic heterogeneity of populations of *Babesia* parasites, as reviewed in section 2.3.1.1, may play an important role in the generation of the immune response and should therefore be considered in immunochemical characterisation studies. Differences between strains of *Babesia* parasites have been demonstrated at the protein composition level (Kahl et al, 1982b; 1983) and at the genetic level (Dalrymple, 1992; Dalrymple et al, 1992). Rapid serial passage of *B.bovis* through splenectomised calves results in its attenuation and this feature of the parasite has been used for production of a live vaccine (Callow & Mellors, 1966). Passage through ticks and *in vitro* cultivation have also been reported to result in changes in parasite populations and their virulence (Callow et al, 1979; Dalrymple et al, 1992). With these considerations in mind, the antigenic differences between the *B.bovis* stocks observed in the present study could have appeared as a consequence of the *in vitro* cultivation of the three original isolates, which might have been antigenically identical or very similar at the time of their first isolation. Cultivation *in vitro* may have acted as an artificial pressure for selection of parasite populations which were more able to survive and multiply continuously *in vitro*. These populations of *in vitro* adapted parasites may differ antigenically from their parent stocks.

A second explanation for the antigenic differences observed between the stocks could be that the original isolates, which initiated the *in vitro* cultured parasite lines, were antigenically different. Previous biochemical analysis of the *B.bovis* stocks at CTVM resulted in a different pattern for GPI isoenzyme from the Lismore stock in comparison to identical patterns for the Mexico and Kwanyanga stocks (T.Melrose, personal communication). The Lismore lysate used for that analysis, however, was produced from infected blood from a calf, whereas the other two stocks were prepared from *in vitro* cultured parasites. Furthermore, when the same comparison was repeated during the present study, using *in vitro* cultured iRBC of each of the three stocks of *B.bovis*, identical patterns were observed (data not shown). Considering that isoenzyme analysis is a useful tool for the discrimination of sub-populations of other protozoans, such as *Theileria annulata* (Melrose & Brown, 1979), these findings may give support to the hypothesis that during *in vitro* cultivation of *Babesia* stocks there is selection of certain populations of parasites, which express different GPI isoenzyme patterns from the majority of the parent stocks.

In the knowledge that antigenic diversity exists amongst the stocks of *B.bovis*, the immunochemical characterisation described here was directed towards the identification of conserved antigens for use as diagnostic tools for the development of species-specific ELISAs.

The study showed that some protein bands detected by immune calf sera were *B.bovis* species-specific and common to all three stocks studied (Table 6.2). They were therefore potential candidates for further analysis. These proteins had molecular weights of 185, 140, 121, 95, 76, 56, 28, 27, 18 and 14 kDa. However amongst those, only some were recognised by the pool of Brazilian field sera. These included the 185, 140, 76, 56 and 14 kDa bands which were identified as the most appropriate candidates for use in the development of *B.bovis* specific ELISAs.

B.bovis antigens with similar molecular weights have been identified by immuno-blotting by other researchers and some of the antigens reported here might be the same or related proteins. Goodger and Wright (1983) identified, amongst others, an antigen of 121 kDa using a saline eluate from sucrose washed bovine RBC infected with *B.bovis*, which might be the same as the 121 kDa antigen identified in the present study. However, their analysis used rabbit antiserum raised against the eluate instead of bovine antiserum. Later, Goodger and co-workers identified four bands with molecular weights of 70, 60, 44 and 27 kDa from a lysate of *B.bovis* iRBC after fractionation by a gel filtration procedure using Sephadex G200 (Goodger et al, 1984). These bands have molecular weights close to some of the bands identified in the present study and may represent the same proteins.

A 29 kDa antigen, which may be the same as the 28 or the 27 kDa proteins identified in the present study, has also been identified to be immunodominant in Western immuno-blotting and has been used for immunisation of susceptible cattle (Wright et al, 1985). It conferred only partial protection against challenge with the homologous strain of *B.bovis*, and was not considered a suitable protective antigen.

B.bovis proteins of molecular weights of 22 and 76 kDa have also been shown to confer partial protection against challenge with the homologous strain and have been considered to be potential protective candidates (Commins et al, 1985). Immunodominant bands of 180 and 140 kDa, from a heparin binding fraction of a *B.bovis* lysate, which might be the same as the 185 and 140 kDa bands identified in the present study, have also been reported to induce partial protection in susceptible cattle against challenge with the homologous strain (Goodger et al, 1987a). Moreover, induction of partial protection has also been reported with proteins of babesial origin which were shown to comprise four immunodominant bands of 12 to 18 kDa in Western immuno-blotting analysis (Goodger et al, 1990). Thus the low molecular

weight bands (14 and 18 kDa) identified in the present study may be the same as those reported by Goodger and co-workers.

The immuno-blotting analysis also allowed the identification of some bands which were faintly recognised by anti-*B.bigemina* sera, and might represent epitopes shared by both *B.bovis* and *B.bigemina*. These were 72, 49 and 23 kDa. It appeared unlikely that these reactions had been generated against RBC components since these bands were not detected when the uninfected control antigen was tested against a pool of anti-*Babesia* sera from calves which had been challenged several times with RBC culture suspensions (Fig 6.6 B). Furthermore one antigen (molecular weight of 200 kDa) was recognised by all sera tested, i.e. *B.bovis* specific, *B.bigemina* specific, field sera and pre-infection sera. This suggests that these reactions may result from cross-reactivity due to similarities between *Babesia* and other agents that the animals had been previously been in contact.

The electrophoretic analysis of *B.bigemina* samples showed, as for *B.bovis*, a large number of bands in both stocks (Kenya and Mexico) by Coomassie blue staining (Fig 6.8). The Western immuno-blotting analysis allowed the identification of major immunodominant bands of the Kenya stock with molecular weights of 94, 80, 79, 71, 65, 60, 59 and 42 kDa (Fig 6.9). However this analysis also indicated great variation in the intensity of reactions between individual calf sera tested, and showed that not all the bands were recognised by all of the serum samples. The strongest reactions were detected with serum from a splenectomised calf (583, taken on day 97 p.i.), which had been inoculated twice with the Zaria stock of *B.bigemina*, and once with the Muguga stock (Fig 6.9, lane 3). The second most intense reactions were observed with serum from an intact calf (397, taken on day 89 p.i.), which had been inoculated twice with the Zaria stock (Fig 6.9, lane 2). On the other hand, the weakest reactions were observed with serum from a splenectomised calf (8T), which had been inoculated twice with the Zaria stock (Fig 6.9, lane 1). Furthermore, the analysis indicated that serum from an intact calf (399) detected only a few bands even after the calf had been inoculated four times with the Zaria stock of *B.bigemina* (Fig 6.9, lanes 4-7). Serum samples from this calf had been shown to contain high titres of specific antibodies in IFAT and ELISA after the first inoculation, which were persistent throughout the study, and no explanation for the low reactivity in Western immuno-blotting was found.

Western immuno-blotting analysis of the Mexico stock of *B.bigemina* resulted in identification of several immunodominant bands (molecular weights ranging from 50 to 255 kDa), as shown in Fig 6.10. Some of them had molecular weights corresponding to those identified in the Kenya stock and were probably the same bands. These included bands at 80, 71 and 65 kDa. The difference in the antigenic patterns of the two stocks of *B.bigemina* may have been due to antigenic differences between them, but may also been resulted from the fact

that each antigen had been prepared by different methods. The Kenya antigen was prepared from infected calf blood and concentrated parasites were sonicated; the Mexico stock antigen was prepared by lysis of concentrated cultured iRBC with SDS-sample buffer. It is possible that each of these methods resulted in a different cleavage of the proteins giving distinct SDS-PAGE separation patterns.

Only one band (molecular weight of 65 kDa) of the *B.bigemina* Kenya stock was faintly recognised by the pool of anti-*B.bovis* sera, which may represent a cross-reactive epitope shared by the two parasites (Fig 6.9, lane 9). However this reaction was very faint when compared to that produced by the anti-*B.bigemina* sera and it was not observed in the *B.bigemina* Mexico stock. The analysis of cross-reactivity using anti-*B.bovis* sera against the Mexico stock of *B.bigemina* showed faintly stained antigens at 71 and 69 kDa (Fig 6.10).

The pool of pre-infection sera did not recognise any of the proteins in the Kenya stock (Fig 6.9, lane 8). However it did recognise faintly two antigens (molecular weights 71 and 69 kDa) in the *B.bigemina* Mexico stock antigen (Fig 6.10), suggesting that these antigens may represent epitopes with similarities to agents other than *Babesia*, with which the calves had been in contact before the *Babesia* infections.

Therefore the summary of the immuno-blotting analysis of somatic antigens of the two stocks of *B.bigemina* (presented in Table 6.3) allowed the identification of two protein bands, which were species-specific and stock-common antigens (molecular weights of 80 and 65 kDa), as potential candidates for use in the development of specific ELISAs. In addition to these, four bands (molecular weights of 195, 162, 128 and 50 kDa), which had been identified as immunodominant in the *B.bigemina* Mexico stock, were also considered as species-specific for further studies, as presented in Chapter 8.

Several specific *B.bigemina* proteins have been identified as potential protective antigens by other research groups. These include proteins with molecular weights of 72, 58, 45 and 36 kDa, which were common to several isolates of *B.bigemina* (McElwain et al, 1987; Figueroa et al, 1990b). However, due to the fact that these studies were based on immunological analysis by immunoprecipitation of radiolabelled parasites rather than by Western immuno-blotting (as reviewed in section 2.3.3.2), it is probably inappropriate to make comparisons with the antigens identified in the present study.

The second approach adopted in the present study was focused on the immunochemical analysis of exoantigens from culture supernatants. This approach unfortunately gave unsatisfactory results which did not allow the identification of species-specific antigens for use in ELISAs.

The presence of soluble antigens in serum from cattle infected with *B.bovis* and *B.bigemina* was first reported by Mahoney (1966). The development of continuous *in vitro* culture systems for both parasites (Levy & Ristic, 1980; Vega et al, 1985a) facilitated studies on the characterisation and dynamics of production of so-called exoantigens, which are defined as soluble proteinaceous moieties naturally released in the plasma of *Babesia* infected animals and in the supernatant of *in vitro* cultures (Ristic & Kakoma, 1988). Biochemical characterisation of *B.bovis* exoantigens has shown that at least three antigens are produced *in vitro* (James et al, 1981), which are associated either with the membrane or cytoplasm of erythrocytes or with the parasite itself (Montenegro-James et al, 1983). Exoantigens of *B.bigemina* from culture supernatant have not yet been immunochemically characterised. Nevertheless, both *B.bovis* and *B.bigemina* culture supernatants have been tested for use as a non-living vaccine in field vaccination trials in some Latin American countries (Smith et al, 1979, Montenegro-James et al, 1985). However, protection conferred to susceptible cattle by culture supernatants has been shown to be only partial after challenge with heterologous strains and to be substantially lower than the protection conferred by live parasites (Timms et al, 1983; 1984).

As the aim of the present study was to identify potential diagnostic target antigens, it was considered that exoantigens from culture supernatant could be a suitable source of antigens which would detect specific antibodies generated against circulating soluble parasite antigens. If such antigens were identified, they could also be used to produce mono-specific antisera (polyclonal or monoclonal), with which to develop an antigen-capture ELISA (Rae & Luckins, 1984; Katende et al, 1990). Assuming that exoantigens are released into plasma of infected animals as soon as the parasite starts to multiply, such an assay would be appropriate for detection of infection at an early stage after primary infection, even before antibodies have been produced. An antigen-capture ELISA could replace the conventional microscopic examination of blood smears for diagnosis of acute infections. Moreover, such an assay could be useful for detection of carrier animals whose antibody levels have dropped and consequently would not be detected as reactive in an antibody detection assay.

The approach used in the present study of applying HPLC to separation of relevant soluble exoantigens, which was based on initial fractionation by size exclusion followed by ion exchange, was chosen with the expectation of isolating antigens from culture supernatant into distinct fractions.

One limitation of the immunochemical analysis of *Babesia* sp culture supernatants appeared to be related to the high proportion of normal serum constituents in the culture medium (40%), which, due to the presence of serum proteins, appeared to mask the antigens of parasite origin. In order to minimise this effect, the proportion of serum in the culture medium

used for harvesting of exoantigens was reduced to 5%, which had been previously determined to have little deleterious effect on parasite growth over a short period (48 hours) (data not shown). However, SDS-PAGE analysis of culture media containing 5% serum showed that a protein band, which was considered to be bovine serum albumin (molecular weight of 66 kDa), still caused distortions in the gel. In an attempt to achieve a better separation of the proteins, the culture supernatants were treated with Affigel blue, which is a cross-linked agarose gel with binding capacity of 11 mg of albumin per ml (Bio-Rad catalogue). This treatment was shown to be effective in removing albumin from the supernatant samples, as demonstrated in Fig 6.12 (lanes 1-4). However, as the Affigel is a suspension of beads in PBS, it results in dilution of the sample after treatment.

Thus, following the removal of albumin, it was necessary to concentrate the culture supernatants. An attempt was made to concentrate them by a filtration procedure using Filtron membranes (Microsep, Microconcentrators - 10 kDa nominal molecular weight cut-off) by centrifugation. According to the manufacturer's recommendations, this method should yield a 100-fold concentration after either 55 minutes of centrifugation at 3,000 x g, 40 minutes at 5,000 x g, or 25 minutes at 7,500 x g. However this was not observed; even after increasing the time of centrifugation up to 5 hours and the speed to 3,000 x g, the volume of supernatant that had been filtered through the membrane was very small with most of the sample being retained by the membrane. After a few minutes of centrifugation the membrane was completely blocked, with a brownish layer being formed around it. This may have happened due to presence of haemoglobin in the samples as a result of lysis of RBC during *in vitro* cultivation. High concentrations of IgG in the culture supernatant may also have contributed to the blocking of the membrane. This method was therefore considered inappropriate for concentration of *Babesia* culture supernatant.

The second method attempted for concentration of albumin-depleted samples was the use of Lyphogel (Gelman), a polyacrylamide hydrogel. In aqueous solutions the lyphogel absorbs 5 times its own weight of water and low-molecular weight substances such as salts; proteins and other substances of a molecular weight of 20 kDa or more are excluded from the gel matrix. Treatment with Lyphogel yielded an approximately five fold concentration of the starting albumin-depleted supernatants (Fig 6.12, lanes 3-6). This method was used throughout the study.

The HPLC fractionation of culture supernatant by size exclusion resulted in similar fractions from both *B.bovis* and uninfected supernatants, in relation to the peaks of absorbances. Similarities in protein composition between infected and uninfected supernatants were also seen by SDS-PAGE analysis (Fig 6.14). The fractionation by size exclusion resulted in elution of small molecular weight proteins first, followed by the heavier ones. This was not

expected since the heavier molecules will pass through the matrix and therefore should be eluted first, whereas the smaller molecules will penetrate the pores of the column matrix and therefore a longer time will be necessary to elute them.

No significant differences were detected in the protein patterns of *B.bovis* and uninfected fractions, although the protein bands in the *B.bovis* fractions stained more intensively than the corresponding bands in the uninfected fractions.

The first Western immuno-blotting analysis of the fractions of *B.bovis* supernatant revealed that two fractions (16 and 17), both had a band of approximate molecular weight of 55 kDa that was recognised by the pool of anti-*B.bovis* sera but not recognised by the pool of pre-infection sera. For this reason these two fractions were selected for further fractionation by ion exchange. However, the Western immuno-blotting analysis of the sub-fractions that originated from the ion exchange fractionation resulted in reaction against the 55 kDa band by both *B.bovis* and pre-infection pools of sera (Fig 6.21). Thus, it was decided to repeat the immuno-blotting analysis using the original samples of the *B.bovis* fractions, including fractions 16 and 17, before they had been fractionated by ion exchange. This analysis resulted in detection of the 55 kDa band in fractions 15 to 18 by both pools of sera. Thus the first detection of the 55 kDa band only by the pool of anti-*B.bovis* sera was not reproducible and none of the bands appeared to be of *B.bovis* origin. In addition to the 55 kDa band, which was reactive in four fractions, one additional reactive band (molecular weight of 120 kDa) was detected in fractions 16 and 17, and one additional reactive band of 80 kDa was detected in fractions 15 and 18 (Fig 6.15). However, all bands appeared to be reactive when either pools of sera (anti-*B.bovis* and pre-infection) were tested and therefore the fractionation of *B.bovis* culture supernatant did not lead to identification of parasite antigens with which to develop specific ELISAs.

The HPLC fractionation of *B.bigemina* culture supernatant also resulted in fractions which had higher absorbances than the corresponding fractions of the uninfected supernatant. These results indicate that some fractions might have contained parasite exoantigens in addition to serum protein components from the NBS used in the culture medium. However the Western immuno-blotting analysis of the fractions from the size exclusion fractionation gave a similar pattern of reactivity to that described for the *B.bovis* supernatant. Two reactive bands (molecular weights of 120 and 55 kDa) were detected when either the pool of anti-*B.bigemina* or the pool of pre-infection sera were tested (Fig 6.24). These bands appeared to be the same as those observed in *B.bovis* supernatant and were not of parasite origin, as demonstrated by the same pattern and intensity of reactions with both pools of sera. Therefore, it was decided not to carry out further fractionation of *B.bigemina* culture supernatant since this approach did not appear to be appropriate for identification of *Babesia* specific exoantigens.

The results obtained from Western immuno-blotting analyses led to the hypothesis that the bands detected from both *B.bovis* and *B.bigemina* supernatant fractions were detected by

reactions from the anti-IgG conjugate to IgG present in the serum used in the culture medium rather than to parasite components. To prove this hypothesis a blot containing samples of normal bovine serum, purified bovine IgG and fractions 16 and 17 of *B.bovis* culture supernatant was reacted with the anti-bovine IgG conjugate without being previously probed with any serum. This analysis resulted in the detection of the same size bands (120 and 55 kDa) in all samples. Therefore it was concluded that the fractions from the HPLC fractionation in fact contained IgG present in culture media.

The whole molecule of bovine IgG has a molecular weight of 150-154 kDa, with the heavy chain representing 54-59 kDa and the light chain 22-24 kDa (Halliwell & Gordon, 1989).

It appears that the 120 kDa band detected in some of the supernatant fractions, which was fainter than the 55 kDa band, represented molecules of IgG which for some reason remained unbroken after the addition of SDS-sample buffer. The 55 kDa band, which was present in all reactive fractions, represented the heavy chains after cleavage by sample buffer, and the light chains (25 kDa) were not detected on the blots, perhaps because they had been blocked during the blocking step, or perhaps due to a lack of specific recognition by the conjugate.

On the other hand, it was possible that specific parasite proteins were present in those fractions in association with IgG and that the IgG molecule may have masked their detection. In an attempt to remove the IgG contamination, fractions 16 and 17 of *B.bovis* supernatant were treated with Protein A, which has high binding affinity to IgG. However immuno-blotting analysis of Protein A-treated fractions showed that although the protein A treatment resulted in a decrease of reactivity against the 55 kDa band, non-specific reactions from the conjugate were still quite strong. Thus, it appears that IgG present in normal bovine serum used for *in vitro* cultivation is a major host contamination and interfered with the identification of specific parasite exoantigens. Removal of bovine IgG should be considered before any fractionation takes place.

The immunochemical analysis presented in this chapter led to the identification of specific somatic proteins of both *B.bovis* and *B.bigemina* with potential use as antigens in immunodiagnostic assays. However, in the face of several limitations regarding the techniques used here, further characterisation of *Babesia* stocks was carried out using immunoprecipitation of radiolabelled proteins as another approach for identification of species-specific components, both somatic and exoantigens. This is presented in the next chapter.

Some of the work reported in this chapter has been published (see Appendix 6.1):

Passos, L.M.F. & Melrose, T.R. (1991) Immunochemical and biochemical diversity in stocks of *Babesia bovis*. *Proceedings of the IV International Congress on Malaria and Babesiosis*, Rio de Janeiro, Brazil (Abstract).

CHAPTER SEVEN

THE USE OF IMMUNOPRECIPITATION TO FURTHER CHARACTERISE STOCKS OF *B.BOVIS* AND *B.BIGEMINA*

7.1 INTRODUCTION

The identification of *B.bovis* and *B.bigemina* specific antigens for use in the development of specific ELISAs was continued using the technique of immunoprecipitation as an alternative method for immunochemical characterisation of cultured parasites. This technique differs from Western immuno-blotting in that the antigens are less completely denatured and thus may retain important conformational epitopes. For this reason, it was believed that immunoprecipitation would enable better use to be made of the McAbs in identification of species-specific antigens.

Immunoprecipitation is a powerful method by which to analyse antigens in complex protein mixtures. The combination of specific antigen-antibody binding and the discrimination by SDS-PAGE separation of proteins by molecular weight permits an impressive resolution of antigenic moieties. The protein mixture of interest is usually labelled with a radioactive amino acid or a precursor of an amino acid by either an exogenous (surface) or endogenous (biosynthetic) labelling technique and, if cell associated, is then solubilised by the addition of a non-ionic detergent such as NP-40. The solubilised antigen mixture is then submitted to high-speed centrifugation to remove insoluble material, and is then reacted with polyclonal or monoclonal antibodies. This results in the formation of an immune complex which is precipitated by the addition of either particles (such as protein A) with binding affinity to IgG, or anti-immunoglobulin antibody. After thorough washing, the immune complex is denatured by boiling in the presence of SDS and its components can be identified by SDS-PAGE separation (Hudson & Hay, 1989).

This chapter presents the results from the immunoprecipitation analysis of ^{35}S-methionine-labelled components of *in vitro* cultures of the three stocks of *B.bovis* (Lismore, Mexico and Kwanyanga), and the Mexican stock of *B.bigemina*. For the identification of species-specific antigens of each parasite, two approaches were taken. One was based on the analysis of soluble parasite components released into the culture supernatant (exoantigens), and the second was based on the analysis of somatic parasite components. In both cases a variety of serum samples were tested, including a panel of calf sera experimentally produced against the different stocks of each parasite, serum samples from cattle naturally infected in the field in Brazil and the panel of anti-*B.bovis* McAbs previously characterised by IFAT, ELISA and Westen immuno-blotting (see Chapter 6).

7.2 MATERIALS AND METHODS

7.2.1 Metabolic labelling with ^{35}S-methionine

The method used was based on that described by Harrison and Parkhouse (1986) for labelling secreted/excreted products of *Taenia saginata*. For each labelling experiment *B.bovis* and *B.bigemina* parasites were cultured in two 2 cm^2 wells of a 24-well plate (1.25 ml/well) as a 10% RBC suspension in methionine-depleted RPMI-1640-ML (as described in Table 3.2). This analysis included all three stocks of *B.bovis* (Lismore, Mexico and Kwanyanga) and *B.bigemina* (Mexico), the only *B.bigemina* stock that could be grown *in vitro*. Each culture was supplemented with L-^{35}S methionine (Amersham) to a final concentration of 50 µCi/ml of culture, for 15-24 hours. Uninfected RBC suspensions from the same donor animals were incubated and prepared under the same conditions as the *Babesia* cultures to provide negative controls.

After incubation, the two wells were pooled and the cells were pelleted by centrifugation at 1,000 x g for 10 min at 4^o C. The supernatant was removed from the cell pellet and centrifuged at 13,000 x g for 30 minutes at 4^o C. The clarified supernatant was then applied to a 1 x 10 cm Sephadex G 25 column (Sigma), previously equilibrated and washed with 50 ml of PBS-methionine (Appendix 1). This gel filtration step was included in order to remove the excess of free ^{35}S-methionine which had not been incorporated by the cells. The supernatant was eluted through the column with PBS and twenty fractions of 0.25 ml were collected. A 10 µl aliquot was taken from every fraction, mixed with scintillation fluid (for aqueous samples, Sigma) and counted in a liquid scintillation analyser (Packard Instruments) to determine the ^{35}S-radioactivity in counts per minute (c.p.m.). Typical c.p.m. of eluted fractions of babesial and uninfected culture supernatants are presented in Appendix 4. Fractions containing incorporated ^{35}S (first peak), which were usually eluted in fractions 5 to 12, were pooled and used as 'exoantigen' in subsequent immunoprecipitation reactions.

The pelleted labelled cells were washed twice in RPMI 1640 and solubilized for 1 h at 4^o C in two volumes of lysis buffer (Appendix 1). The resultant extracts were centrifuged at 13,000 x g for 30 min at 4^o C to remove insoluble material. The supernatant from this lysate was used as 'somatic antigen' in subsequent immunoprecipitations.

To estimate the incorporation of ^{35}S-methionine after each labelling, samples of exoantigen and somatic antigen were precipitated with trichloroacetic acid (TCA). Aliquots of 10 µl of each sample were mixed with 190 µl of NBS and 2 ml of 10% (w/v) TCA and allowed to stand for 10 minutes at room temperature. The resultant precipitates were washed twice by centrifugation at 3,000 x g for 5 minutes with NaOH, resuspended in 4 ml of scintillation fluid and the c.p.m. measured (as previously described). The percentage of ^{35}S-methionine

incorporation was calculated by subtracting the c.p.m. obtained after precipitation from the initial (total) c.p.m.. Typical percentages of incorporated ^{35}S in exoantigens and somatic antigens from babesial and uninfected cultures are presented in Appendix 4.

7.2.2 Immunoprecipitation

In order to minimise non-specific background, labelled parasite antigens (exo- and somatic) were treated with serum before being used in immunoprecipitations. For this, a mixture of 200 µl of either normal bovine (in the case of precipitation with polyclonal sera) or normal mouse (in the case of precipitation with McAbs) serum, 1.0 ml of co-precipitation diluent (Appendix 1) and 400 µl of rabbit anti bovine or goat anti mouse Ig (Sigma), as appropriate, was left overnight at 4° C to allow non-specific precipitation. One hundred µl of this suspension were mixed with 100 µl of ^{35}S-labelled parasite extract (either exo- or somatic antigens) and the mixtures were centrifuged at 13,000 x g for 5 min at 4° C. The radioactivity of resultant supernatants (in c.p.m.) was estimated by scintillation counting as previously described and the supernants (designated 'clean probe') were stored at -80° C until required.

The panel of serum samples used throughout the analysis included calf sera produced at CTVM by experimental inoculation of RBC infected with each parasite species and stock (as described in Table 3.1), calf sera produced in Brazil by experimental inoculation of infected blood, and serum samples collected from cattle which had been exposed to natural infestation with ticks in Brazil (as described in section 3.4.2). A pool of pre-infection sera was also included as a negative control and was prepared by mixing equal volumes of samples taken from calves 396, 397, 399 and 583 before infection with *Babesia* parasites. In some experiments pools of anti-*B.bovis* and anti-*B.bigemina* sera were used. The pool of anti-*B.bovis* sera was prepared by mixing equal volumes of serum samples taken from calves 396 (days 57 and 104), P78 (day 80) and 198 (day 28); the pool of anti-*B.bigemina* sera contained serum samples from calves 397 (day 69), 399 (day 98), 583 (day 85) and 8T (day 28). In an attempt to reduce non-specific background reactions these pools of sera were pre-adsorbed with normal RBC prior to being used in immunoprecipitations.

The anti-*B.bovis* McAbs used are described in section 3.4.3, and results of their characterisation by IFAT, ELISA and Western immuno-blotting are presented in Chapter 6.

Duplicate aliquots of 5 µl of each polyclonal serum or McAb under investigation were incubated with a volume of each clean probe containing 5 x 10^4 c.p.m. at 4° C for 1 hour. One hundred and fifty µl of anti-bovine or anti-mouse Ig diluted 1:10 with PBS were added, and the mixtures were incubated at 4° C overnight to allow precipitation. The precipitates

were washed 4 times by centrifugation (3,000 x g for 10 min at 4° C) in ice-cold co-precipitation diluent and resuspended in 25 µl of PBS. An aliquot of 5 µl was removed for scintillation counting and the remaining 20 µl was mixed with 10 µl of either reducing or non-reducing electrophoresis sample buffer (Appendix 1). Samples for SDS-PAGE (total proteins and precipitates) were boiled for 5 minutes, centrifuged for 3 minutes (at 10,000 x g) to remove insoluble material and run on either 7-20% gradient or 10% homogeneous gels (as described in section 3.6.2). Radioactive (^{14}C) standard molecular weight markers (Amersham) were loaded in at least one lane of each gel to enable calculation of the relative mobility of the proteins. In addition, a sample of the corresponding clean probe (total protein) was loaded in each gel.

7.2.3 Fluorography and autoradiography

After electrophoresis, gels containing ^{35}S-radiolabelled samples (total proteins and/or immune complexes) were fixed for 30 minutes in a 10% (v/v) methanol, 5% (v/v) acetic acid solution and then washed for 2 x 30 minutes in DMSO (Sigma). Gels were then transferred sequentially into the following solutions: a 27.5% (w/v) 2,5-Diphenyloxazole (BDH)/DMSO solution for 3 hours; tap water overnight; and a 1% (v/v) glycerol, 10% (v/v) acetic acid solution for 1 hour. The processed gels were dried under vacuum using a Bio-Rad apparatus (model 224). Dried gels were then exposed to X ray films (Agfa-Curix) using fast tungstate intensifying screens (Ilford) in metallic cassettes, and were stored at -80°C up to 6 weeks, depending on the radioactivity of the samples (e.g. gels containing samples with counts of 2,000 c.p.m. were exposed for 4 weeks). After the appropriate exposure time the cassettes were allowed to thaw at room temperature over a period of 2 hours and the films were developed in a dark room. Films were soaked in developer solution (Sigma) for 4 minutes and in fixative solution (Sigma) for 4 minutes. After being rinsed in tap water, films were allowed to dry and were photographed through filter F61 as described in Appendix 3.

7.3 *B.BOVIS* SOMATIC ANTIGENS

7.3.1 Total protein profiles

At least 35 protein bands with molecular weights ranging from 21 to 200 kDa were detected in total protein samples of *B.bovis* ^{35}S-labelled lysates, whereas no bands were detected in samples of lysates from uninfected cultures, as shown in Fig 7.1. More numerous and clearly resolved bands were seen in samples treated with reducing buffer (Fig 7.1, lanes 1, 3 and 5) than in samples treated with non-reducing buffer (lanes 2, 4 and 6).

The pattern of protein separation was very similar amongst the three stocks analysed. However the Lismore stock differed from the Mexico and Kwanyanga stocks with regard to two bands. The former lacked a protein band (105 kDa) that was seen in the Mexico and Kwanyanga stocks; while a distinct protein band of 195 kDa was detected only in the Lismore stock (Fig 7.1, arrows on the left).

7.3.2 Antigen profiles

When the total lysate of *B.bovis* (Lismore stock) was immunoprecipitated with anti-*B.bovis* sera, many bands were detected. Several of these were also detected by the pool of pre-infection and anti-*B.bigemina* sera, as shown in Fig 7.2. The pattern of protein separation obtained with reducing and non-reducing buffers was similar, except in relation to a few protein bands. The molecular weights of bands were calculated based on the pattern obtained under reducing conditions (rc).

The antigenic profile of the Lismore stock showed that five bands were *B.bovis* specific (precipitated by anti-*B.bovis* sera, but not by pre-infection or anti-*B.bigemina* sera). These were located at 88, 75, 65, 41 and 24 kDa (Fig 7.2).

Four bands with molecular weights corresponding to those detected in the Lismore stock, were also identified in the Mexico stock, and, in addition to these, one extra *B.bovis* specific antigenic band (46 kDa) was identified in the Mexico stock (Fig 7.3).

Five protein bands with molecular weights corresponding to those identified in the Mexico stock (88, 75, 65, 46 and 41 kDa) were also seen in the Kwanyanga stock (Fig 7.4). In addition to these, two extra bands (26 and 24 kDa) were identified as *B.bovis* specific in the Kwanyanga stock.

Thus, results from this analysis allow the identification of four somatic antigenic components (88, 75, 65 and 41 kDa) as being *B.bovis* specific and stock-conserved antigens.

The immunoprecipitation of somatic antigen using the panel of McAbs showed that four of the 11 McAbs precipitated proteins in the Mexico stock. Three of them (1B8:1, 118.67.2 and 4F3:1) precipitated a 240 kDa protein (Fig 7.5, lanes 4, 13 and 14). In addition to the 240 kDa protein, McAb 1B8:1 precipitated a 49 kDa protein, and McAb 118.67.2 precipitated a 27 kDa protein. McAb 3.D6.1 precipitated a single protein of 36 kDa (Fig 7.5, lane 11). However when the panel of McAbs was tested against the Lismore and Kwanyanga lysates, no protein bands were detected, as shown in Fig 7.6 A and B.

Fig 7.1 SDS-PAGE of ^{35}S-labelled proteins of *B.bovis* lysates.

Lanes: 1 and 2 are Lismore stock lysate

3 and 4 are Mexico stock lysate

5 and 6 are Kwanyanga stock lysate

In 1, 3 and 5 samples were treated with reducing sample buffer; in 2, 4 and 6 samples were treated with non-reducing sample buffer.

Lane 7 contains lysate of a ^{35}S-labelled uninfected RBC culture (negative control).

Standard molecular weight markers are indicated on the right.

Fig 7.1

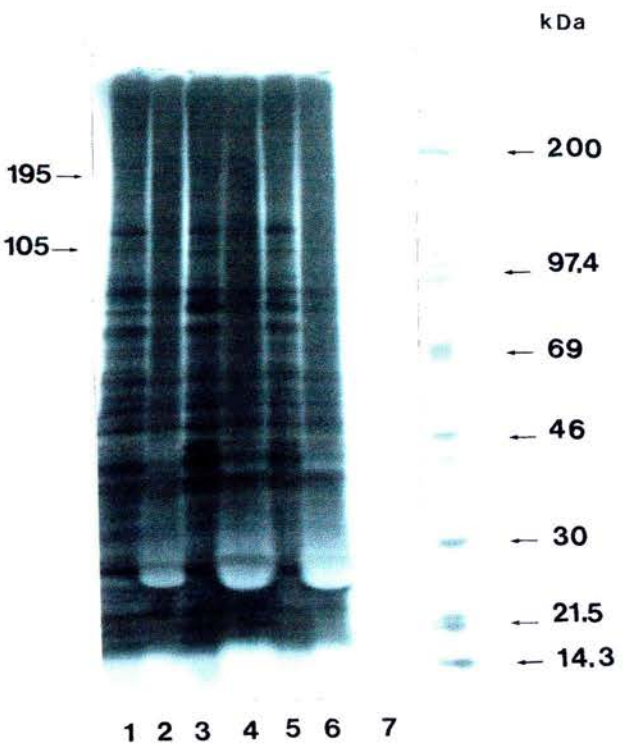


Fig 7.2 Immunoprecipitation of somatic antigens of the Lismore stock of *B.bovis* with polyclonal sera.

rc= reducing conditions, nc= non-reducing conditions

Lanes: 1 - total protein (rc)

2 - precipitation (pp) with a pool of anti-*B.bovis* sera (CTVM) (rc)

3 - as in 2 (nc)

4 - pp with a pool of anti-*B.bovis* sera (Brazil) (rc)

5 - as in 4 (nc)

6 - pp with a pool of pre-infection sera (rc)

7 - as in 6 (nc)

8 - pp with a pool of anti-*B.bigemina* sera (rc)

9 - as in 8 (nc)

10- total protein (nc)

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate *B.bovis* specific bands.

Fig 7.2

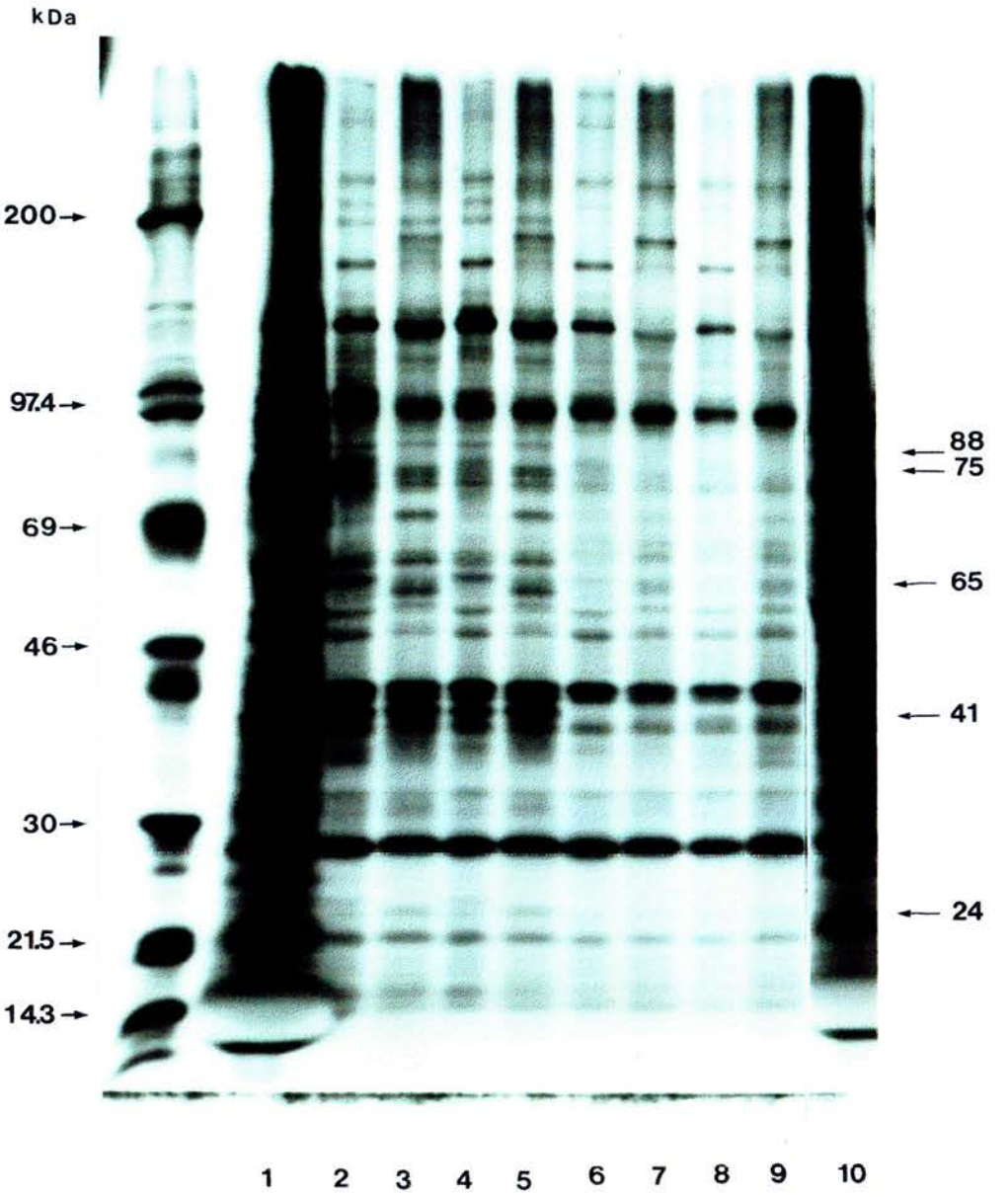


Fig 7.3 Immunoprecipitation of somatic antigens of the Mexico stock of *B.bovis* with polyclonal sera.

Lanes: 1 - total protein (rc)

2 - total protein (nc)

3 - precipitation (pp) with a pool of anti-*B.bovis* sera (CTVM) (rc)

4 - as in 3 (nc)

5 - pp with a pool of anti-*B.bovis* sera (Brazil) (rc)

6 - as in 4 (nr)

7 - pp with a pool of pre-infection sera (rc)

8 - as in 6 (nc)

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate *B.bovis* specific bands.

Fig 7.3

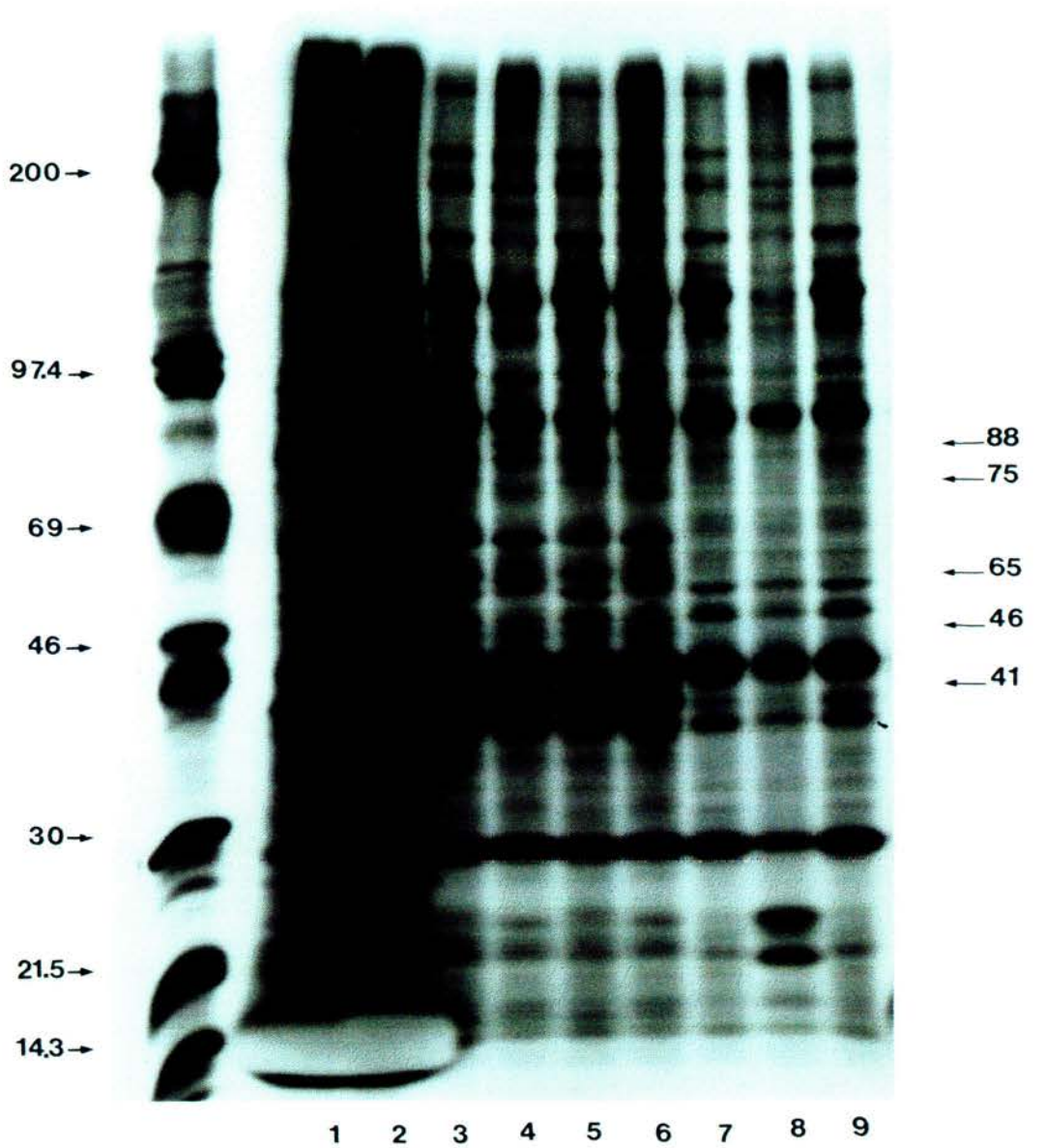


Fig 7.4 Immunoprecipitation of somatic antigens of the Kwanyanga stock with polyclonal sera

Lanes: 1 - total protein (rc)

2 - total protein (nc)

3 - precipitation (pp) with a pool of anti-*B.bovis* sera (CTVM) (rc)

4 - as in 3 (nc)

5 - pp with a pool of anti-*B.bovis* sera (Brazil) (rc)

6 - as in 4 (nc)

7 - pp with a pool of pre-infection sera (rc)

8 - as in 6 (nc)

9 - pp with a pool of ant-*B.bigemina* sera (rc)

10- as in 9 but (nc)

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate *B.bovis* specific bands.

Fig 7.4

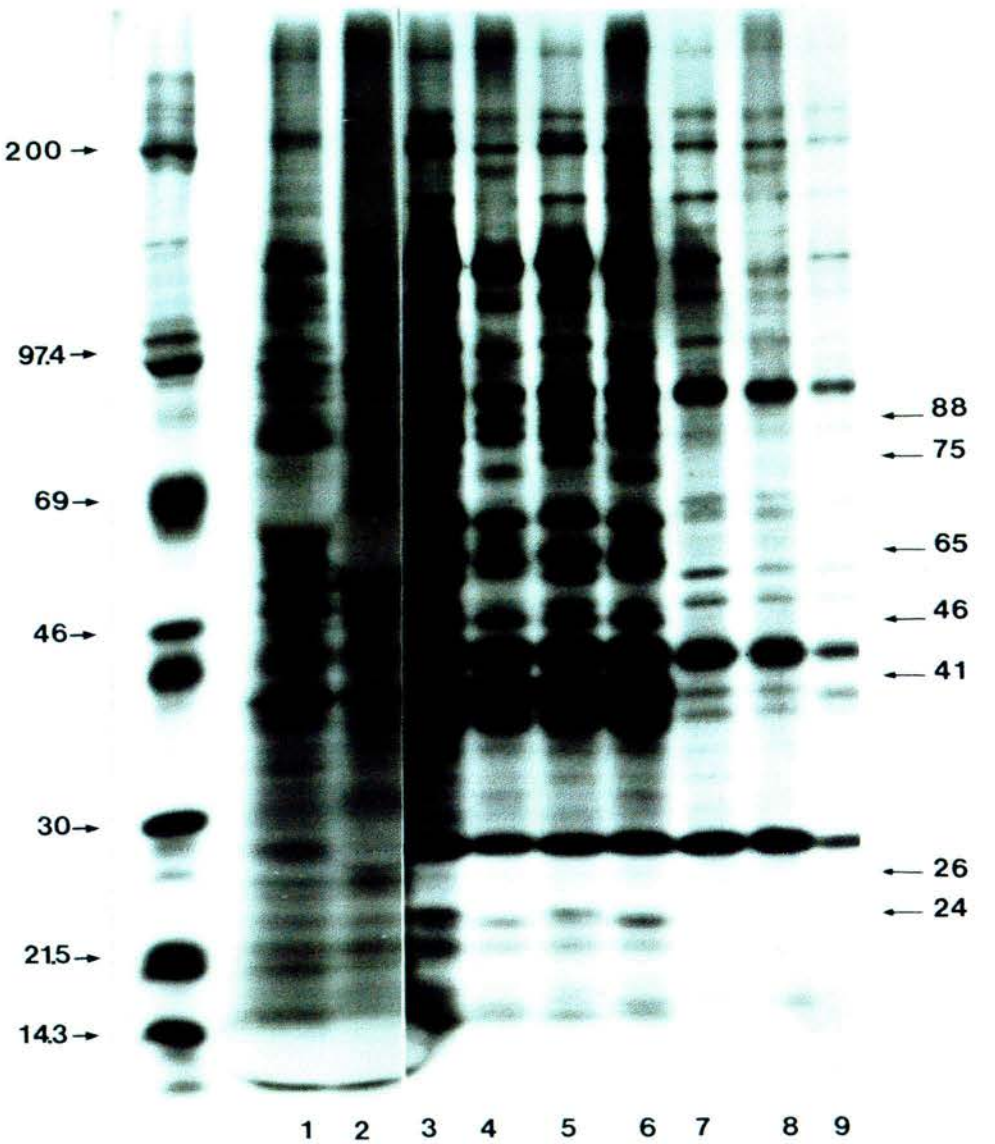


Fig 7.5 Immunoprecipitation of somatic antigens of the Mexico stock of *B.bovis* with McAbs

Lanes: 1 - total protein

2 - PBS control

3 - anti-*Trypanosoma* McAb (negative control)

4 - McAb 1B8:1

5 - McAb 2C8:2

6 - McAb 1C12:1

7 - McAb 4D1:3

8 - McAb 1C12:2

9 - McAb 3D4:2

10-McAb 5G12

11-McAb 3D6.1

12-McAb 2A11

13-McAb 118.67.2

14-McAb 4F3:1

Standard molecular weight markers are indicated on the right.

Arrows on the left indicate precipitated bands.

Fig 7.5

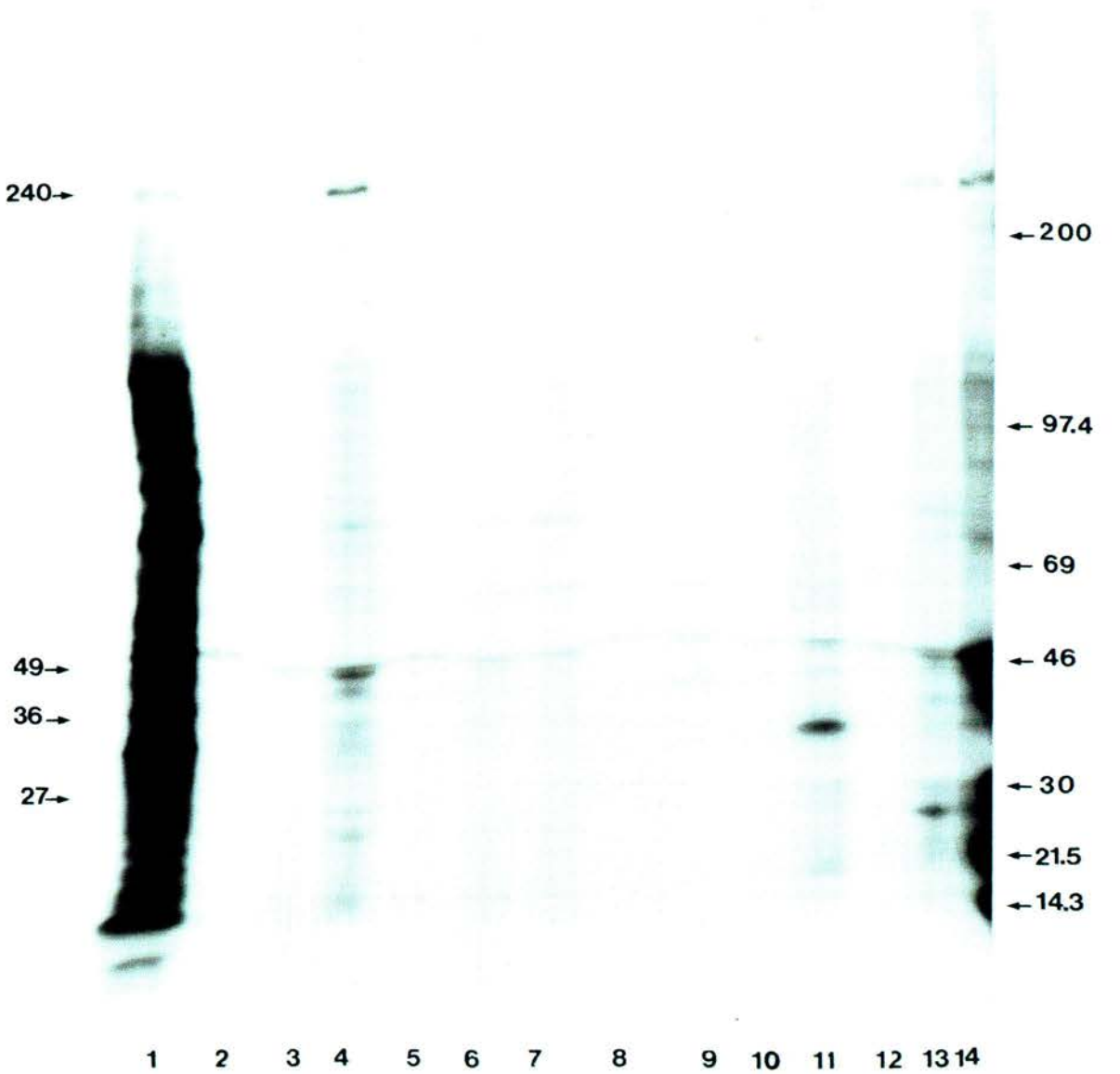


Fig 7.6 Immunoprecipitation of somatic antigens of the Lismore (A) and the Kwanyanga stocks (B) with McAbs

Lanes: 1 - total protein

2 - anti-*Trypanosoma* McAb (negative control)

3 - McAb 1B8:1

4 - McAb 2C8:2

5 - McAb 1C12:1

6 - McAb 4D1:3

7 - McAb 1C12:2

8 - McAb 3D4:2

9 - McAb 5G12

10-McAb 3D6.1

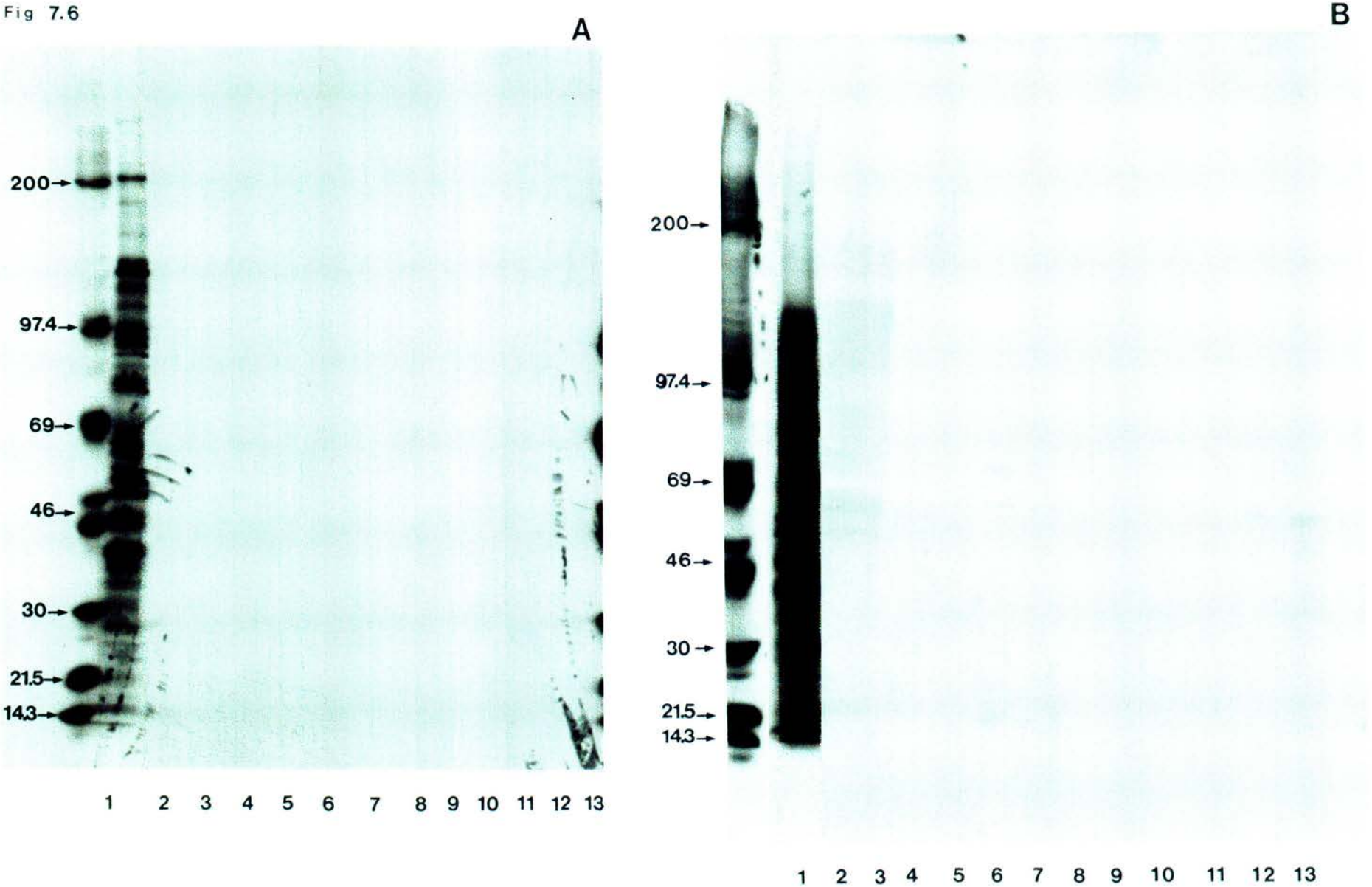
11-McAb 2A11

12-McAb 118.67.2

13-McAb 4F3:1

Standard molecular weight markers are indicated on the left.

Fig 7.6


A
200
97.4
69
46
30
21.5
14.3
1 2 3 4 5 6 7 8 9 10 11 12 13
B
200
97.4
69
46
30
21.5
14.3
1 2 3 4 5 6 7 8 9 10 11 12 13

7.4 *B.BOVIS* EXOANTIGENS

7.4.1 Total protein profiles

Approximately 28 bands of labelled proteins (with molecular weights ranging from 200 to 14 kDa) were detected in culture supernatants of *B.bovis*, whereas no bands were detected in the uninfected RBC culture supernatant (negative control), as shown in Fig 7.7. The pattern of protein profiles was very similar between the three stocks, except in relation to a few proteins. Two bands (200 and 185 kDa) that were clearly detected in the Kwanyanga stock were not detected in the Lismore and Mexico stocks, and a band at 160 kDa was present only in the Lismore stock (Fig 7.7, arrows). In addition, four low molecular weight bands (26, 25, 23 and 21.5 kDa) were seen in the supernatants of Mexico and Kwanyanga.

7.4.2 Antigen profiles

The profile of exoantigens from the Lismore stock obtained after immunoprecipitation with polyclonal sera is presented in Fig 7.8. Many of the protein bands resulting from immunoprecipitation with homologous *B.bovis* antisera (Fig 7.8, lanes 4-9) were also present, although with less intensity, with both heterologous *B.bigemina* antisera (Fig 7.8, lane 2) and pre-infection sera (Fig 7.8, lane 3). Five bands, which were not clearly resolved, were detected in immunoprecipitates from anti-*B.bovis* sera but not detected, or only very faintly detected, in immunoprecipitates from both anti-*B.bigemina* and pre-infection sera. These were seen at molecular weights 250, 169, 80, 29 and 22 kDa, as shown in Fig 7.8 (arrows).

The profile of exoantigens of the Mexico stock of *B.bovis* revealed that at least 8 bands were strongly precipitated by anti-*B.bovis* sera and not precipitated by either anti-*B.bigemina* or pre-infection sera (Fig 7.9). These were seen at molecular weights 80, 79, 72, 58, 38, 34, 24 and 22 kDa, as indicated by arrows in Fig 7.9.

The immunoprecipitation analysis of the Kwanyanga stock exoantigens resulted in a profile similar to that observed with the Mexico stock. Ten antigens, many with molecular weights corresponding to those identified as *B.bovis* specific in the Mexico stock culture supernatant (80, 72, 58, 38, 34, 24 and 22 kDa) were also immunoprecipitated in the Kwanyanga culture supernatant (Fig 7.10). In addition to these, three extra antigens were detected as *B.bovis* specific in the Kwanyanga supernatant. These were seen at molecular weights 120, 44 and 42 kDa.

When immunoprecipitates from culture supernatants of each of the *B.bovis* stocks were separated on the same gel, 6 antigens were identified as *B.bovis* specific and common to the three stocks analysed (Fig 7.11). These were seen at molecular weights 80, 72, 58, 38, 34 and

24 kDa, and therefore these were identified as exoantigens which were *B.bovis* specific and present in all three stocks.

None of the McAbs immunoprecipitated any of the exoantigens of the Lismore and Kwanyanga stocks of *B.bovis*. However two faint bands at 120 and 40 kDa were seen in precipitates of each McAb tested against the Mexico stock, as shown in Fig 7.12.

Fig 7.7 Total protein profiles of ^{35}S-labelled supernatants of *B.bovis* and uninfected RBC cultures.

Lanes: 1 - Lismore stock

2 - Mexico stock

3 - Kwanyanga stock

4 - uninfected RBC

Standard molecular weight markers are indicated on the right.

Arrows on the left indicate differences in protein profiles between stocks.

Fig 7.7

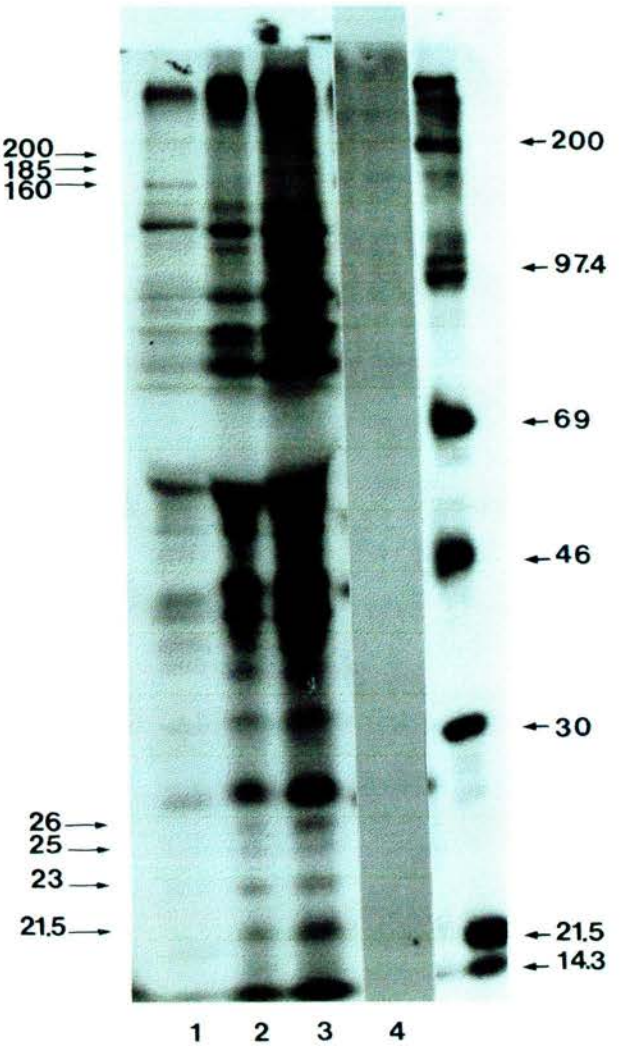


Fig 7.8 Immunoprecipitation of exoantigens of the Lismore stock of *B.bovis* using pooled polyclonal sera.

Lanes: 1 - total exoantigens

2 - precipitation (pp) with a pool of anti-*B.bigemina* sera

3 - pp with a pool of pre-infection sera

4 - pp with calf 396 serum (day 104)

5 - pp with calf P78 serum (day 96)

6 - pp with calf P78 serum (day 42)

7 - pp with calf P78 serum (day 21)

8 - pp with calf C11 serum (day 28)

9 - pp with calf 198 serum (day 28)

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate *B.bovis* specific bands.

Fig 7.8

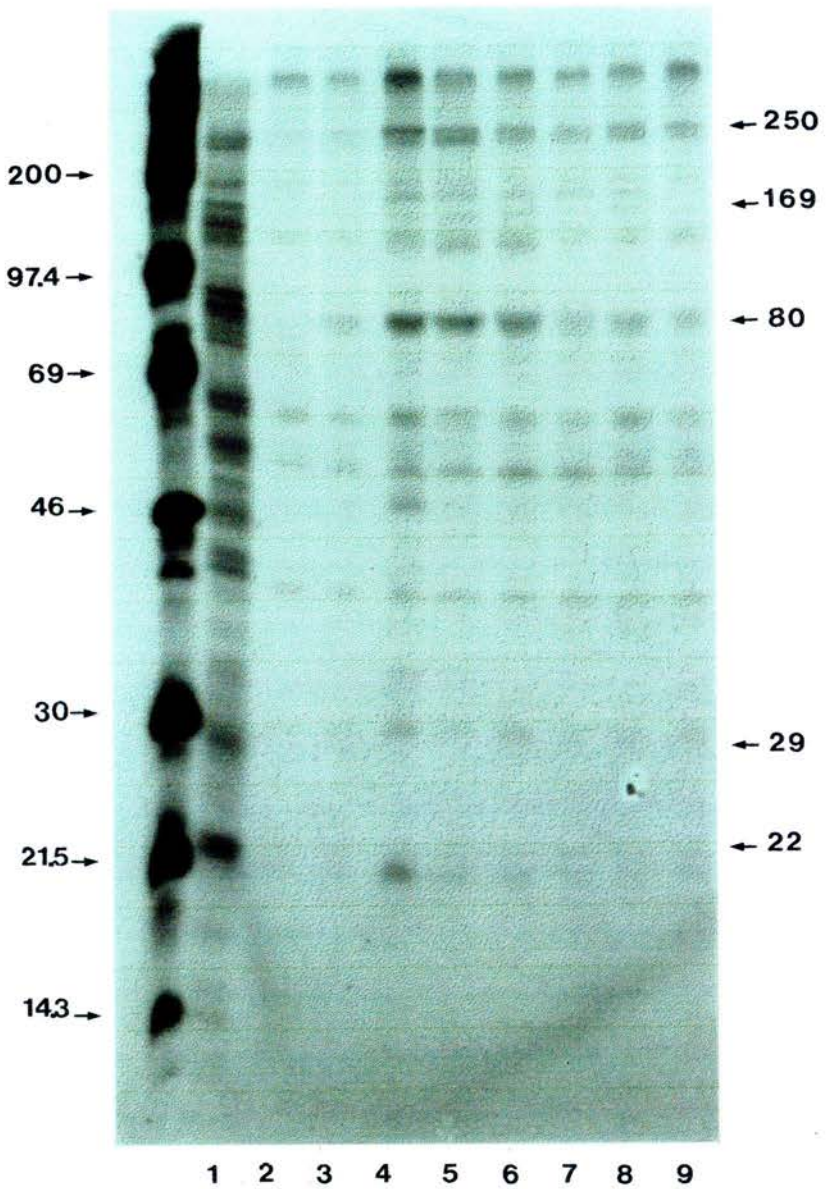


Fig 7.9 Immunoprecipitation of exoantigens of the Mexico stock of *B.bovis* using polyclonal sera.

Lanes: 1 - pp with a pool of anti-*B.bovis* sera

2 - pp with a pool of pre-infection sera

3 - pp with a pool of anti-*B.bigemina* sera

4 - pp with distilled water

5 - pp with foetal calf serum

6 - pp with a pool of calf P78 sera (days 21, 42and 96)

7 - pp with calf 396 serum (day 28)

8 - pp with calf 396 serum (day 56)

9 - pp with calf 396 serum (day 84)

10-pp with calf 396 serum (day 104)

11-pp with a pool of anti-*B.bovis* (Brazilian) sera

12-total exoantigens

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate *B.bovis* specific bands.

Fig 7.9

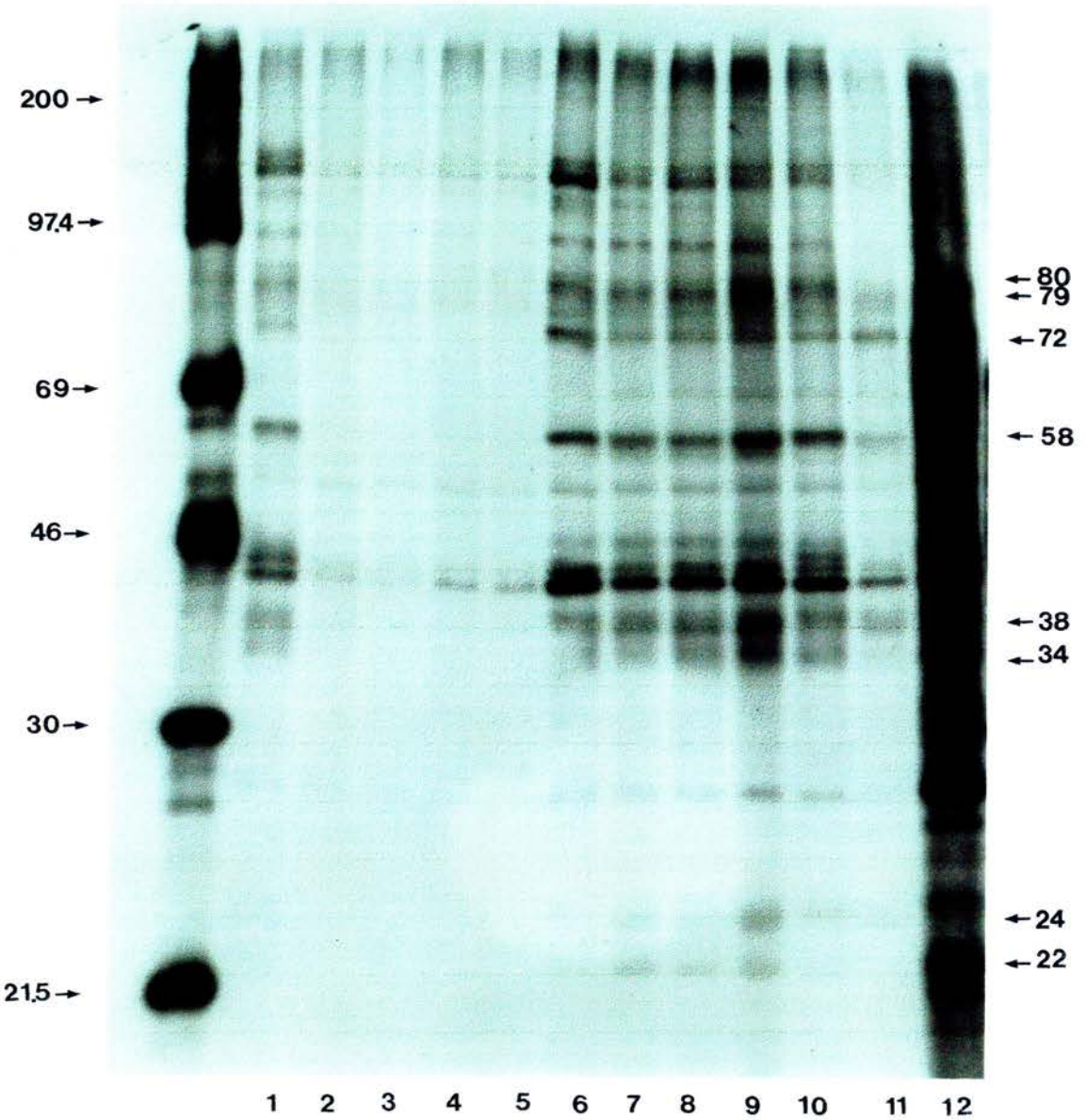


Fig 7.10 Immunoprecipitation of exoantigens of the Kwanyanga stock of *B.bovis* using polyclonal sera.

Lanes: 1 - pp with a pool of anti-*B.bovis* sera

2 - pp with a pool of pre-infection sera

3 - pp with a pool of anti-*B.bigemina* sera

4 - pp with distilled water

5 - pp with a pool of calves 198 and C11 sera (day 28)

6 - pp with calf P78 serum (day 21)

7 - pp with calf P78 serum (day 42)

8 - pp with calf P78 serum (day 96)

9 - pp with calf 396 serum (day 104)

10-pp with a pool of anti-*B.bovis* (Brazilian) sera

11-total exoantigens

Standard molecular weight markers are indicated on the right.

Arrows on the left indicate *B.bovis* specific bands.

Fig 7.10

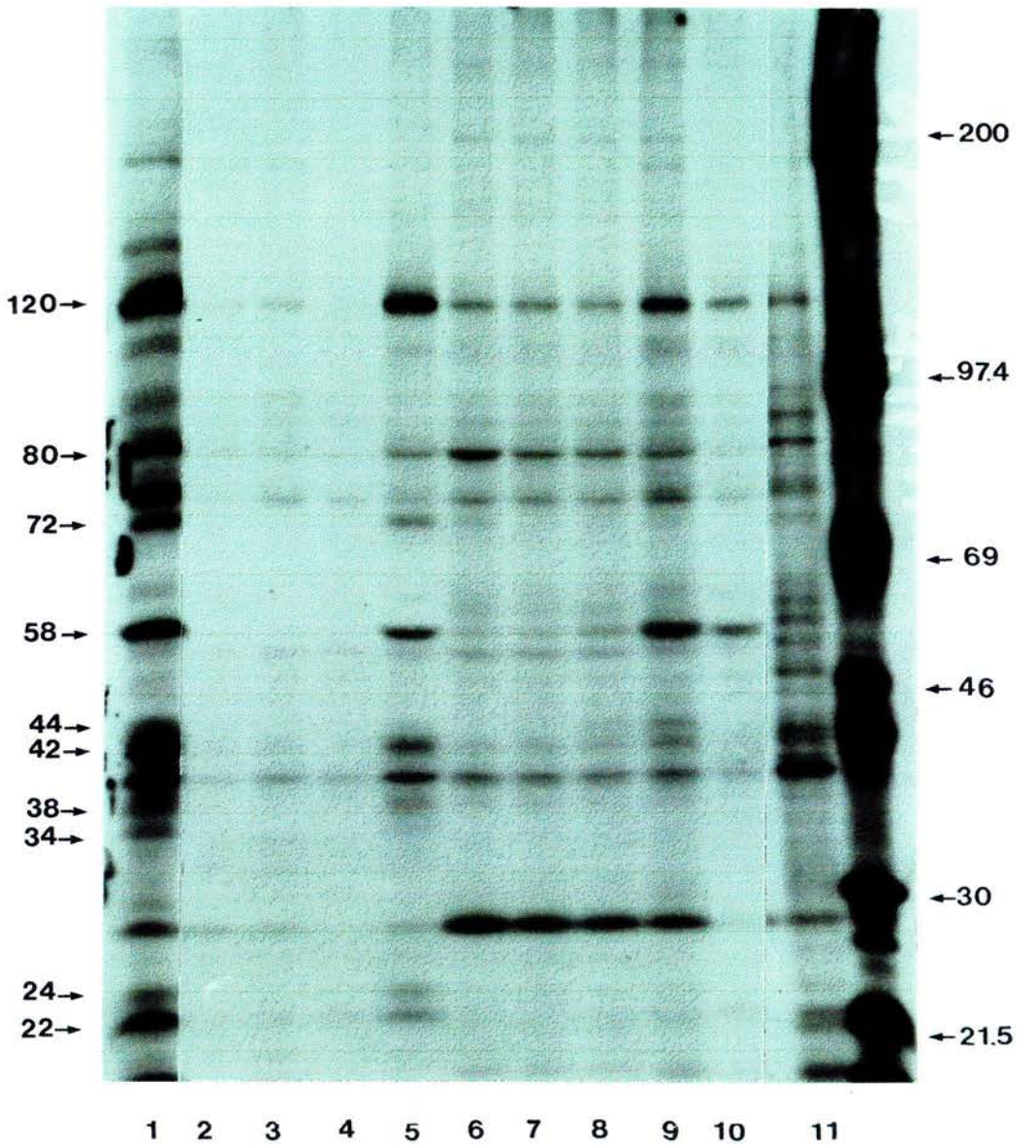


Fig 7.11 Immunoprecipitation of exoantigens of stocks of *B.bovis* using polyclonal sera.
Lanes 1, 4, 7 and 10 are Lismore stock; lanes 2, 5, 8 and 11 are Mexico stock; lanes 3, 6, 9 and 12 are Kwanyanga stock.
A shows pp with a pool of anti-*B.bovis* sera
B shows pp with a pool of Brazilian field sera
C shows pp with a pool of anti-*B.bigemina* sera
D shows pp with a pool of pre-infection sera.
Standard molecular weight markers are indicated on the left.
Arrows on the right indicate *B.bovis* specific bands.

Fig 7.11

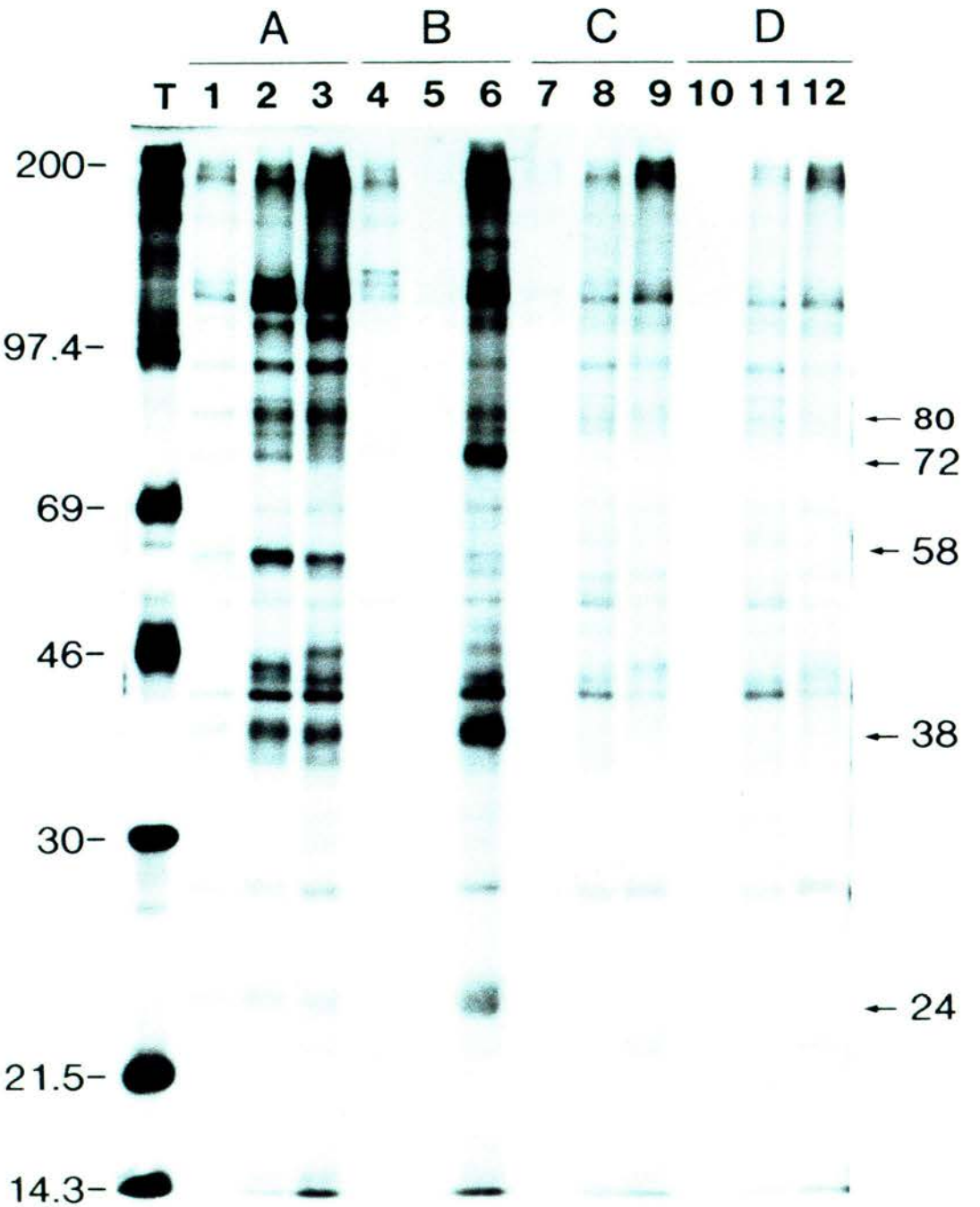


Fig 7.12 Immunoprecipitation of *B.bovis* (Kwanyanga stock) exoantigens using McAbs.

Lanes: 1 - pp with McAb 1B8:1

2 - pp with McAb 2C8:2

3 - pp with McAb 1C12:1

4 - pp with McAb 4D1:3

5 - pp with McAb 1C12:2

6 - pp with McAb 3D4:2

7 - pp with McAb 5G12

8 - pp with McAb 3D6.1

9 - pp with McAb 2A11

10-pp with McAb 118.67.2

11-pp with McAb 4F31

12-pp with McAb PBS

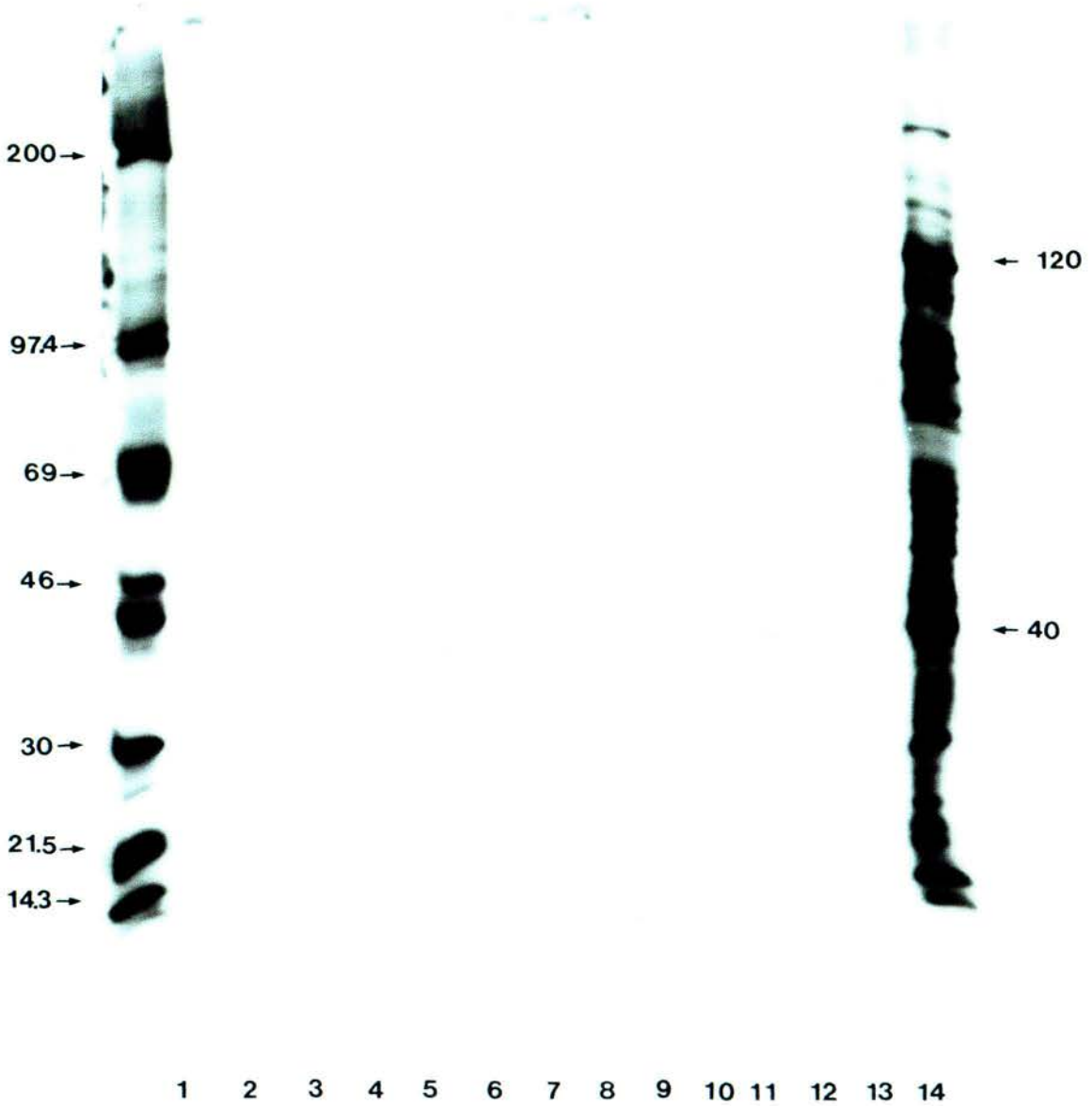
13-pp with McAb anti-*Trypanosoma*

14-total exoantigens

Standard molecular weight markers are indicated on the left.

Arrows on the left indicate the two faint bands seen with all McAbs.

Fig 7.12



7.5 *B.BIGEMINA* ANTIGENS

7.5.1 Total protein profiles

Approximately 40 labelled protein bands (with molecular weights ranging from 200 to 14 kDa) were seen in the lysate of *B.bigemina* (Mexico stock), as shown in Fig 7.13, A; and at least 23 bands were detected in the culture supernatant (Fig 7.13, B).

7.5.2 Somatic antigen profiles

At least 30 bands were seen in precipitates resulting from the reaction between *B.bigemina* lysate and anti-*B.bigemina* sera (Fig 7.14, lanes 2-11). However many of these were also seen in precipitates using either pre-infection sera (Fig 7.14, lane 1) or anti-*B.bovis* sera (Fig 7.14, lane 13). Twelve bands were detected in precipitates of homologous sera and were not detected in precipitates from either pre-infection or anti-*B.bovis* sera. These were seen within the range from 210 to 21 kDa and are indicated by arrows in Fig 7.14.

7.5.3 Exoantigen profiles

Immunoprecipitation analysis of exoantigens of the Mexico stock of *B.bigemina* revealed that four bands were precipitated by anti-*B.bigemina* sera but not by pre-infection sera (Fig 7.15). These were seen at molecular weights 240, 112, 50 and 29 kDa as indicated by arrows in Fig 7.15. These bands were not seen in precipitates using anti-*B.bovis* sera (data not shown) and therefore were identified as *B.bigemina* specific exoantigens.

Fig 7.13 SDS-PAGE of ^{35}S-labelled proteins of *B.bigemina* (Mexico stock).
Panel A contains a lysate of iRBC and panel B contains culture supernatant.
In A the standard molecular weight markers are indicated on the left and in B on the right.

Fig 7.13

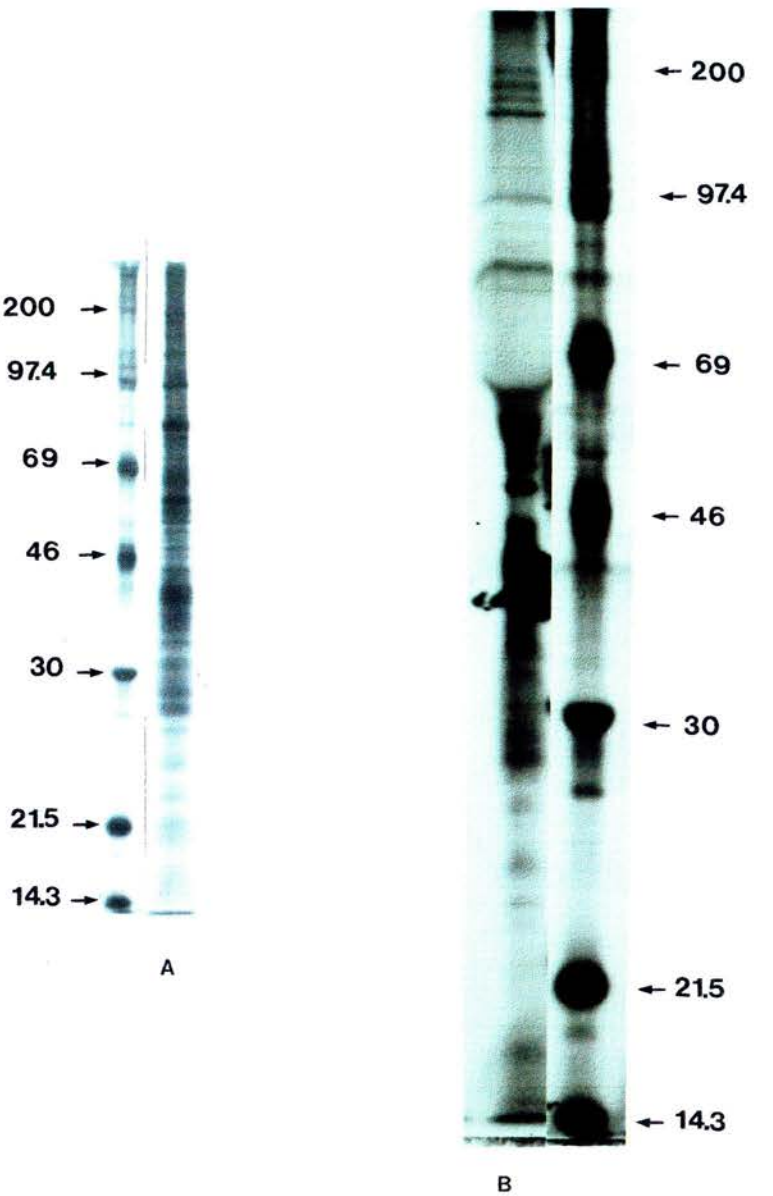


Fig 7.14 Immunoprecipitation of somatic antigens of *B.bigemina* (Mexico stock) using polyclonal sera.

Lanes: 1 - pp with a pool of pre-infection sera

2 - pp with calf 399 serum (day 29)

3 - pp with calf 399 serum (day 71)

4 - pp with calf 399 serum (day 119)

5 - pp with calf 397 serum (day 28)

6 - pp with calf 397 serum (day 89)

7 - pp with calf 583 serum (day 28)

8 - pp with calf 583 serum (day 64)

9 - pp with calf 583 serum (day 97)

10-pp with a pool of anti-*B.bigemina* sera (CTVM)

11-pp with a pool of anti-*B.bigemina* sera (Brazil)

12-pp with a pool of Brazilian field sera

13-pp with a pool of anti-*B.bovis* sera

Standard molecular weight markers are indicated on the left and right.

Arrows on the left point to specific somatic antigens of *B.bigemina* and ascribe molecular weights.

Fig 7.14

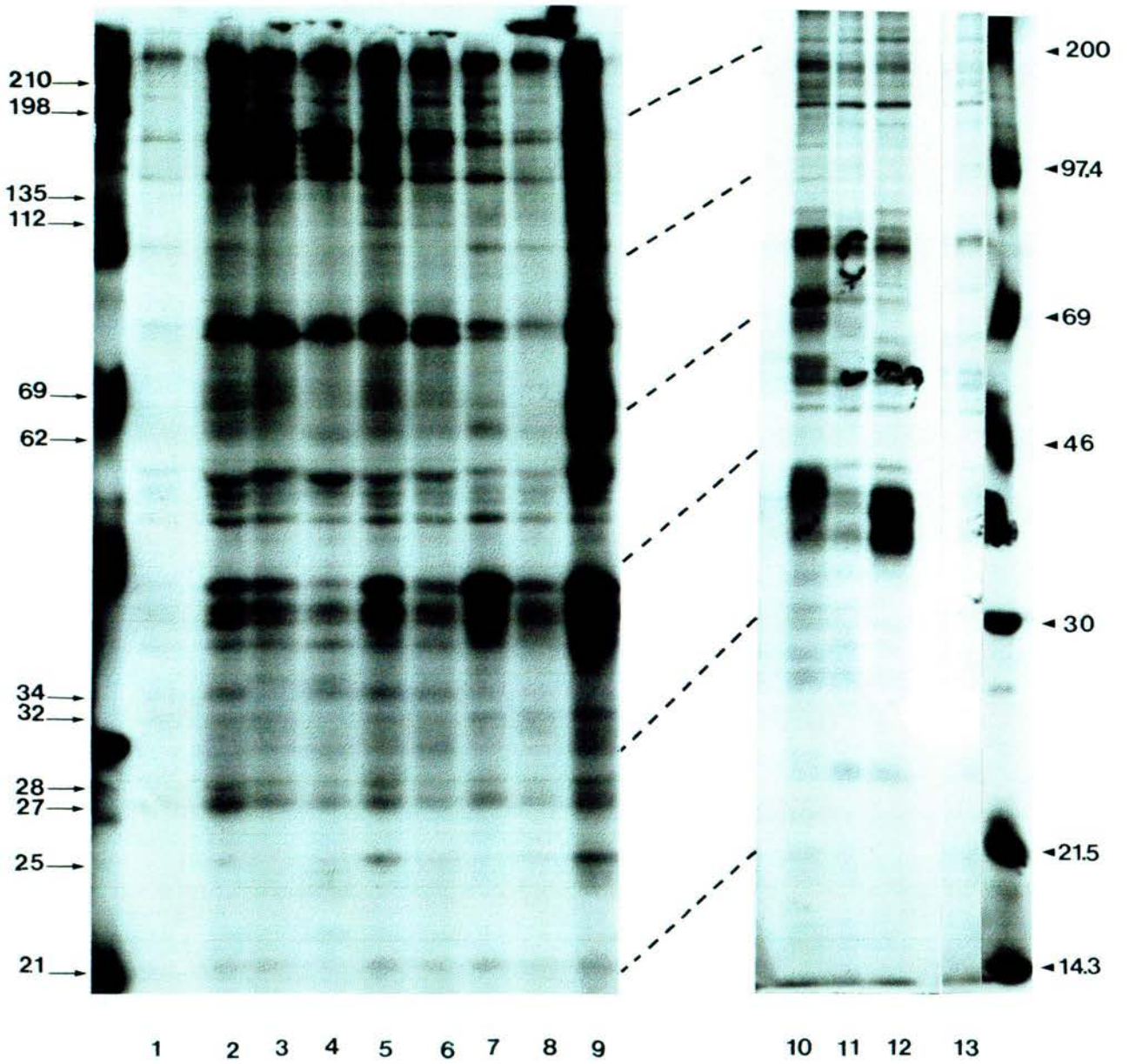

210
198
135
112
69
62
34
32
28
27
25
21
200
97.4
69
46
30
21.5
14.3
1
2
3
4
5
6
7
8
9
10
11
12
13

Fig 7.15 Immunoprecipitation of exoantigens of *B.bigemina* (Mexico stock) using polyclonal sera.

Lanes: 1 - total exoantigens

2 - pp with a pool of pre-infection sera

3 - pp with calf 399 serum (day 29)

4 - pp with calf 399 serum (day 71)

5 - pp with calf 397 serum (day 87)

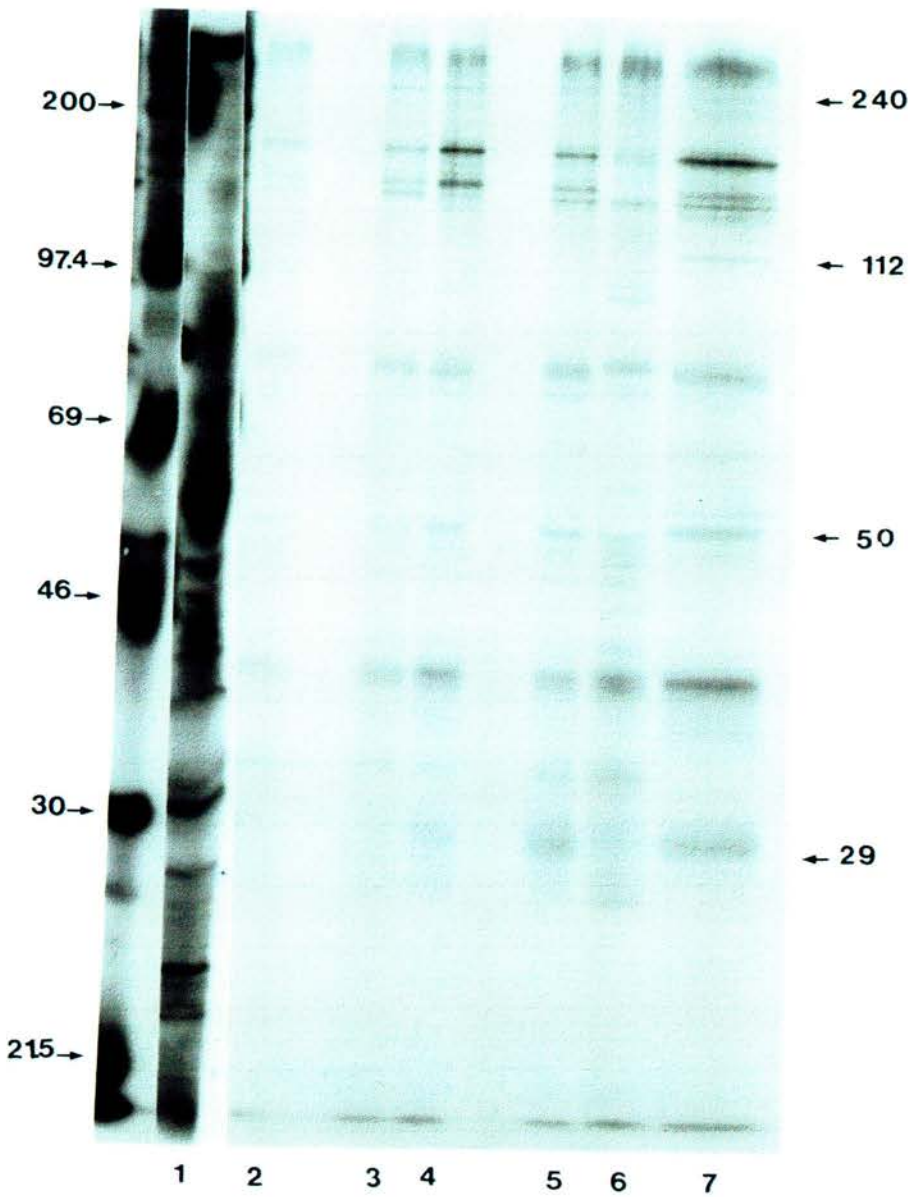
6 - pp with calf 583 serum (day 28)

7 - pp with calf 583 serum (day 97)

Standard molecular weight markers are indicated on the left.

Arrows on the right indicate specific exoantigens of *B.bigemina*.

Fig 7.15



7.6 DISCUSSION AND CONCLUSIONS

The results presented in this chapter show that immunoprecipitation of ^{35}S-labelled components is an appropriate tool for the identification of babesial antigens. This technique was found to be very sensitive and allowed the identification of several *B.bovis* and *B.bigemina* antigens in both somatic and extracellular components from culture supernatants.

Proteins metabolically labelled with ^{35}S-methionine were detected in both lysates and supernatants of *Babesia* infected cultures but not in lysates or supernatants of uninfected cultured RBC, proving that the components labelled were of parasite origin. The level of incorporation of ^{35}S-methionine by iRBC was consistently high in all labelling experiments, with an average of 70% and 90% precipitatable proteins (TCA) in somatic components and exoantigens respectively (Appendix 4A and B). These indicate that the culture conditions used for the metabolic labelling were adequate and allowed good parasite metabolism.

Although measures were taken to preclear the labelled protein mixtures prior to adding specific antibodies, high background was observed in the SDS gels throughout the analysis. Background problems may come from several different sources and can be either specific as when antigens are recognised directly by spurious antibodies or non-specific occurring due to contaminant proteins that either bind to particulate matter in the preparation or aggregate and are precipitated with the immune complex (Harlow & Lane, 1988). In the present study the observed background appeared to be non-specific. This was inferred because similar background reactions were observed when distilled water or PBS were used instead of serum to precipitate labelled proteins, and also by unsuccessful attempts to minimise background by pre-incubating the test sera with normal RBC. In order to minimise the non-specific background, an alternative protocol was attempted in which protein A was used to precipitate the immune complex. However this was found to be no better than the co-precipitation protocol with similar non-specific background still being detected with pre-infection sera and PBS. In spite of this non-specific background, the inclusion of negative controls (pre-infection sera and PBS or distilled water) throughout the analysis permitted the identification of specific antigens without difficulty.

As expected, separation of immune complexes under reducing conditions resulted in better resolution and a greater number of protein bands than under non-reducing conditions (ilustrated in Fig 7.2-7.4). This was probably due to complete cleavage of polypeptides by the reducing agent mercaptoethanol present in the reducing sample buffer (Hames & Rickwood, 1990). Thus the subsequent identification of target antigens was based on separation under reducing conditions.

The present study showed antigenic diversity between the three stocks of *B.bovis* at the two levels analysed (somatic and exoantigens). As discussed in Chapter 6, where antigenic diversity between the stocks was also indicated by Western immuno-blotting, this may be related to specific differences in the protein profiles amongst the stocks, which originated from different isolates. Thus, the present analysis was focused on the identification of stock-common components which could be used as target antigens for the development of specific ELISAs. The results obtained here confirm that, although the Mexico and the Kwanyanga stocks differed in some proteins, their protein profiles showed greater similarity to each other than to that of the Lismore stock. In immunoprecipitation, as in Western immuno-blotting, the Brazilian field sera reacted more strongly against antigens of the Kwanyanga and Mexico stocks than against those of the Lismore stock. This supports the hypothesis, presented in Chapter 6, that Brazilian isolates of *B.bovis* may be more antigenically similar to the Kwanyanga stock than to the Lismore; however, in contrast to the Western immuno-blotting results, immunoprecipitation suggested that the Brazilian isolates also had similarities to the Mexico stock of *B.bovis*.

Four *B.bovis* somatic antigens fulfilled the criteria of being species-specific, present in all three stocks, and recognised by both experimentally raised sera and field sera in immunoprecipitations. These antigens had molecular weights of 88, 75, 65 and 46 kDa and were identified as potential candidates for further analysis. Other workers have identified somatic *B.bovis* proteins by immunoprecipitation of labelled components. These include a 42 kDa protein which was found to be species-specific and stock-conserved (McElwain et al, 1988) and a 44 kDa merozoite surface protein (Reduker et al, 1989). The 46 kDa protein identified in the present study might be the same as one of these.

The *B.bovis* somatic proteins identified here had different molecular weights from those identified by Western immuno-blotting (Chapter 6) and only the 75 kDa might be the same as the 76 kDa protein previously identified as species-specific and conserved amongst stocks in Chapter 6. The identification of different proteins in the present study was probably related to differences in the two techniques used. Western immuno-blotting detects antigens which have been denatured by the action of boiling with SDS-reducing buffer, whereas in immunoprecipitation the antigen mixture is reacted with the serum without previous denaturation of the polypeptides (Harlow & Lane, 1988). Furthermore, the immunoprecipitation detects labelled components which might be present in minute amounts and therefore may not be detected by Western immuno-blotting. Thus, antigens resulting from immunoprecipitation are not expected to be the same as those detected by Western immuno-blotting.

The panel of McAbs which had been raised against *B.bovis* (Kwanyanga) was included in this analysis in the hope of being able to use them in an affinity column procedure to purify specific antigens from a crude mixture of *B.bovis* components. In the present analysis four McAbs precipitated proteins in the Mexico stock lysate. These were seen at molecular weights 200, 49, 36 and 27 (Fig 7.5). The four reactive McAbs had previously been found to recognise internal parasite components by IFAT and three of them were also reactive in ELISA (as described in Chapter 6). These findings suggest that McAbs 1B8:1, 118.67.2 and 4F3:1 recognise *B.bovis* epitopes which may be related to internal parasite components.

Despite reacting in IFAT against all three stocks of *B.bovis*, none of the McAbs immunoprecipitated proteins in the Lismore and Kwanyanga stocks and therefore the approach of using them in an affinity purification column was not pursued.

The somatic antigens which were thus considered to be the most promising potential candidates for the development of *B.bovis* specific ELISAs were those identified by polyclonal sera in Western immuno-blotting (molecular weights ranging from 185 to 14 kDa, as described in Chapter 6) and immunoprecipitation (88, 75, 65 and 46 kDa, described in the present chapter). As affinity column purification could not be used, an alternative method of purification of the proteins of interest was attempted by electro-elution of protein bands from acrylamide gels. To use this approach it was necessary to visualise the bands of interest needed to be visualised by conventional staining methods to enable their excision from the gel. Coomassie blue staining of acrylamide gels containing total lysates of *B.bovis* showed well defined protein bands at molecular weights corresponding to four candidate proteins (121, 75, 56 and 46 kDa). Thus, these were selected for the development of *B.bovis* specific ELISAs (Chapter 8).

The analysis of *B.bigemina* somatic antigens was limited to the only stock (Mexico) that could be grown *in vitro*. As observed for the three *B.bovis* stocks, a wide range of labelled proteins was detected in the total lysate of *B.bigemina*. High non-specific background was observed and the more intense bands detected in precipitates from anti-*B.bigemina* sera were also seen in precipitates from anti-*B.bovis* and pre-infection sera. However several weak bands were detected as being *B.bigemina* specific antigens (Fig 7.14). Similar protein profiles were seen with serum samples taken from three calves infected with *B.bigemina* (Zaria stock) regardless of the length of time after primary infection and number of challenges. In contrast, serum taken from a fourth calf (583) showed much more intense bands after challenge with the Muguga stock in comparison to the antigenic profiles obtained from sera taken after challenges with the Zaria stock (Fig 7.14, lanes 9 and 8). This suggests that the Mexico and Muguga stocks may have more antigenic similarities than the Mexico and Zaria stocks of

B.bigemina. However, as 583 was the only calf challenged with the Muguga stock it was not possible to make any conclusive comparison between stock-specific sera.

The precipitated proteins identified as *B.bigemina* specific antigens were minor components and none of them had molecular weights corresponding to those previously identified by Western immuno-blotting (Chapter 6). The identification of *B.bigemina* proteins by immunoprecipitation of ^{35}S-labelled components using McAbs has been reported by other authors. These include several proteins (molecular weights ranging from 72 to 36) found to be located on the surface of merozoites (McElwain et al, 1987; Figueroa et al, 1990a). More recent studies have been focused on the characterisation and synthesis of a 58 kDa protein (Mishra et al, 1991) and its use as an immunogen alone or in combination with a high molecular weight (> 200 kDa) protein, also identified by McAbs in immunoprecipitation of ^{35}S-labelled components (McElwain et al, 1992). The latter protein has been shown to be associated with the membrane of infected erythrocytes (McElwain et al, 1992) and may be the same as the 210 kDa protein identified in the present study.

As the present study did not have access to McAbs against *B.bigemina* for use in an immunoaffinity purification, the candidate somatic proteins for the development of *B.bigemina* specific ELISAs were those identified by polyclonal sera in either Western immuno-blotting (molecular weights ranging from 195 to 50 kDa, as described in Chapter 6) or immunoprecipitation, described in the present chapter (Fig 7.14). The next step to determine the applicability of these antigens in a diagnostic test was to purify the *B.bigemina* proteins by electro-elution from acrylamide gels and use them in *B.bigemina* specific ELISAs. Coomassie blue staining of acrylamide gels containing total *B.bigemina* lysate detected four well-defined bands with molecular weights corresponding to some of the candidate proteins (210, 80, 65 and 50). These were then selected for use in the development of *B.bigemina* specific ELISAs.

In the search for exoantigens, a wide range of protein bands were detected in labelled supernatants of both *B.bovis* and *B.bigemina* cultures. It was assumed that these were secreted or excreted parasite components, as no labelled proteins were detected in supernatant of uninfected cultures. Several species-specific proteins were identified in both *B.bovis* and *B.bigemina* labelled culture supernatants. However, in order to utilise these proteins it was necessary to purify parasite components from culture supernatant and this emerged as a factor limiting further progress in the present study. Due to the use of high proportion of NBS in *Babesia* culture media a large number of protein bands are seen in acrylamide gels making it difficult to identify bands that are of parasite origin. It was expected that HPLC fractionation of culture supernatants would enable separation of the complex mixture of proteins into fractions containing certain ranges of molecular weight components, which

would facilitate location of parasite components in acrylamide gels. However this approach was not found to be appropriate for purification of *Babesia* exoantigens from culture supernatants (Chapter 6). The electro-elution of exoantigens from acrylamide gels was not found to be an appropriate approach either, since the parasite components, which were present in minor amounts, were masked by protein components present in high concentrations in the NBS. Thus, the methods attempted for purification of exoantigens did not allow purification of supernatant components and for this reason it was not possible to use any of the exoantigens identified as candidate antigens in the development of species-specific ELISAs (Chapter 8). Perhaps better results would be achieved if, prior to attempts to purify exoantigens, most of the contaminating serum components were removed by an affinity column. In addition, it appeared from results of the present analysis that in order to have clear visualisation of *Babesia* exoantigens in acrylamide gels, culture supernatants would need to be concentrated considerably (Chapter 6). Exoantigens from culture supernatant have been purified and characterised for *P.falciparum* (Thelu et al, 1985), used in experimental vaccination against *B.divergens* (Valentin et al, 1993), and form the basis of the commercial vaccine "Pirodog" (Merieux) against *B.canis* (Molinar et al, 1982). Although it was hoped that exoantigens would play a major role in the development of discriminatory diagnostic assays for bovine babesiosis, the lack of success in purifying exoantigens from cultures points to the need for improvement and adaptation of the methods used in the present study, and the need for development of other methods for purification of babesial exoantigens from culture supernatants.

Some of the work reported in this chapter has been published (see Appendix 6.2):

Passos, L.M.F. (1993) Identification of immunodominant *Babesia bovis* exoantigens. *Trans R Soc Trop Med Hyg* 87, 122.

CHAPTER EIGHT

DEVELOPMENT OF ELISAs FOR DETECTION OF ANTIBODIES AGAINST *B.BOVIS* AND *B.BIGEMINA*

8.1 INTRODUCTION

Due to difficulties in the detection of *Babesia* parasites in the blood of animals with chronic infections using conventional methods, epidemiological investigations are usually based on results from immunodiagnostic techniques designed to detect antibodies against *Babesia* species. These methods have been reviewed in section 2.2.2. Each assay has both advantages and disadvantages depending on its sensitivity, specificity, simplicity and the costs involved.

The ELISA system is considered to be a very sensitive and economical assay, ideal for use in epidemiological surveys where a large number of samples need to be tested. Since its first application for detection of antibodies against *Babesia* species (Purnell et al, 1976) the aim has been primarily to improve the quality of reagents in order to obtain more reliable tests for epidemiological studies in the field. Particular problems with *Babesia* arise from the fact that the parasite develops almost exclusively inside erythrocytes, making it difficult to obtain pure parasite antigen preparations. Crude antigens contain host components, such as erythrocyte stroma and membrane, which result in non-specific antibody binding. These non-specific reactions usually contribute to high background absorbance values in sera from animals which have not been infected with *Babesia* parasites (Bose et al, 1990).

In order to improve the quality of antigen preparations, the aim has been to identify parasite fragments or soluble exoantigens that react with specific antibodies. However, due to similarities between species of *Babesia*, cross-reactions are commonly observed in most serological tests. Cross-reactivity between *B.bovis* and *B.bigemina* becomes a problem in discrimination of the two infections in endemic areas where they usually occur in association, having a common vector in *B.microplus*. Furthermore, antigenic diversity among different strains of *B.bovis* has also been described (Kahl et al, 1982b; Kahl et al, 1983; McElwain et al, 1988) and these antigenic differences between isolates may have important roles in determining the extent and specificity of the immune response. Such factors must be considered in any attempt to select parasite components to be used as antigens for diagnostic or immunogenic purposes.

Thus, work has been directed towards the identification of specific antigens of *B.bovis* and *B.bigemina* for use as diagnostic tools.

Through the immunochemical characterisation of stocks of *B.bovis* and *B.bigemina* described in Chapters 6 and 7, several candidate proteins were identified that might be of use as antigens for immunodiagnostic purposes.

This chapter describes attempts to develop specific ELISAs based on the use of these proteins as antigens for species-specific antibody detection. The proteins selected for this purpose had been shown to be species-specific when analysed by other immunological techniques as

described in previous chapters. The approach used here was based on the elution of the proteins of interest from acrylamide gels containing crude parasite antigen preparations.

The assays using eluted parasite proteins were developed on a comparative basis with assays using crude antigen preparations of each *Babesia* species as reference.

8.2 ELISAS USING CRUDE PARASITE ANTIGENS

8.2.1 Materials and methods

8.2.1.1 Crude antigen preparations

A crude *B.bovis* (Lismore stock) antigen was used as the reference preparation. This had been prepared from culture suspensions with a parasitaemia of 7% by Woodford and co-workers (Woodford et al, 1990). Briefly, iRBC from culture suspensions (as described in 3.3.2) were concentrated by differential hypotonic lysis with KCl and lysed with NH_4Cl, as described for SDS-PAGE samples in 3.6.1.1. The final lysate was then sonicated for 3 x 30 seconds at maximum amplitude in a 100-W ultrasonic disintegrator (MSE, Crawley, U.K.), and the supernatant obtained after centrifugation at 10,000 x g for 5 minutes was stored as 100 µl aliquots at -80° C until required for ELISAs.

A crude *B.bigemina* antigen lysate which had been produced at the ILRAD, Kenya, from *B.bigemina* (Kenya stock) was used as a reference antigen. Parasites had been obtained from blood taken from a calf during ascending *B.bigemina* parasitaemia, concentrated after cryopreservation in DMSO, and disintegrated by sonication (as described in 6.5.1, J. Katende, personal communication). This antigen was received at CTVM as 100 µl aliquots frozen in liquid nitrogen and was stored at -80°C until required for ELISAs.

8.2.1.2 Standardisation of assays

The ELISA procedure used throughout this study was as described in section 3.8.

The crude *B.bovis* antigen was used at a dilution of 1 in 1,000 and test sera were diluted 1 in 100. These dilutions had been judged as optimal after checkerboard titration against standard control sera (Woodford et al, 1990).

Throughout the study the anti-bovine IgG peroxidase conjugate (Sigma) was used at a dilution of 1 in 6,000, according to the manufacturer's recommendation.

The crude *B.bigemina* antigen was used at a dilution of 1 in 1,000 and test sera were diluted 1 in 100, based on a checkerboard titration previously carried out at ILRAD (J.Katende, personal communication).

Each crude antigen was tested against a panel of calf sera of known provenance, including 15 samples taken before infection, 13 samples taken at specific periods after infection with *B.bovis*, and 19 samples taken at specific periods after *B.bigemina* infection (details of calf infections are given in Table 3.1).

All serum samples were tested in duplicate and their means calculated. The standard deviation (SD) of mean readings from the group of negative sera was calculated and used for the calculation of positive-negative discrimination levels (cut-off values), according to Richardson and co-workers (Richardson et al, 1983). Absorbance values higher than the mean of negative sera plus twice their SD were considered as positive, while values lower were considered as negative.

The sensitivity of each assay was defined as the proportion of known positive serum samples detected as positive and the specificity as the proportion of known negative serum samples detected as negative.

8.2.2 Results

8.2.2.1 *B.bovis* assay

The absorbance values obtained from each pair of wells tested against the panel of calf serum samples are presented in Appendix 5.1 and are summarised in Table 8.1. The mean absorbance value from the anti-*B.bovis* serum group was approximately 2.8 times greater than that obtained with pre-infection sera. An anti-*B.bovis*:anti-*B.bigemina* absorbance ratio of 2.2:1 was obtained with the *B.bovis* crude antigen assay. The greatest variation in absorbance values was observed amongst anti-*B.bovis* sera (SD = 0.190), whereas the groups of pre-infection and anti-*B.bigemina* sera showed lower variations (SD = 0.061 and 0.080, respectively).

The calculated cut-off point was 0.310. This gave the assay a sensitivity of 0.85, since only 11 of the 13 known anti-*B.bovis* sera were detected as positive (Table 8.2). All 15 samples of pre-infection sera were negative. Two of the 19 anti-*B.bigemina* sera were detected as positive for *B.bovis*, giving the assay a specificity of 0.94.

The absorbance readings obtained from the anti-*B.bovis* sera were distributed over a wide range of values (Fig 8.1 A); whereas absorbance values obtained from anti-*B.bigemina* and pre-infection sera were each distributed in a more limited range, resembling a normal distribution curve (Fig 8.1 B and C, respectively).

Table 8.1 Absorbance values in experimentally raised sera and pre-infection sera tested against crude *B.bovis* antigen in ELISA.

Sera (No of samples)	Mean	Standard deviation	No of positives/ total
anti-*B.bovis* (13)	0.522	0.190	11/13
anti-*B.bigemina*(19)	0.233	0.080	2/19
pre-infection (15)	0.188	0.061	0/15

Table 8.2 Sensitivity and specificity of ELISA using crude *B.bovis* antigen.

Status of samples	Proportion
True positives (sensitivity)	0.85 (11/13)
True negatives (specificity)	0.94 (32/34)
False positives	0.06 (2/34)
False negatives	0.15 (2/13)

Fig. 8.1 Frequency distribution of absorbance values obtained with experimentally raised sera and pre-infection sera tested against *B.bovis* crude antigen in ELISA.

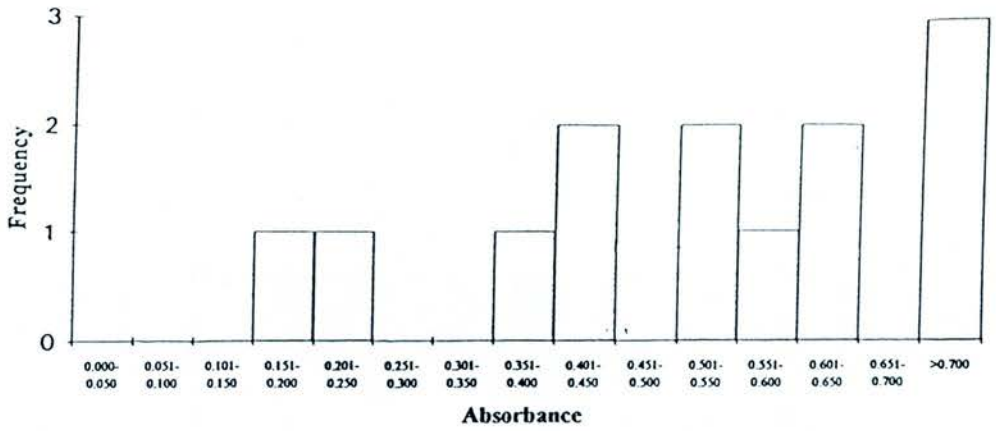
A is anti-*B.bovis* sera

B is anti-*B.bigemina* sera

C is pre-infection sera

A

Fig 8.1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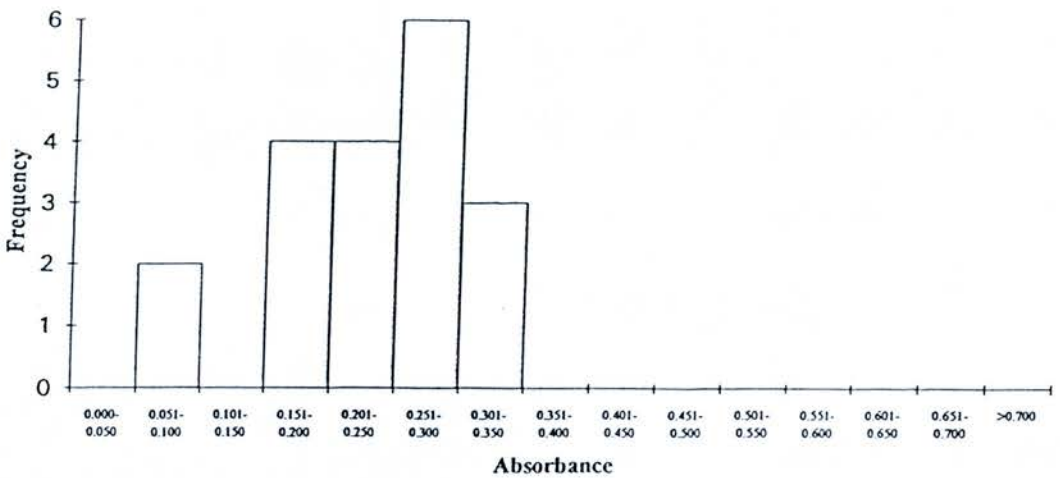
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
>0.700
Absorbance

B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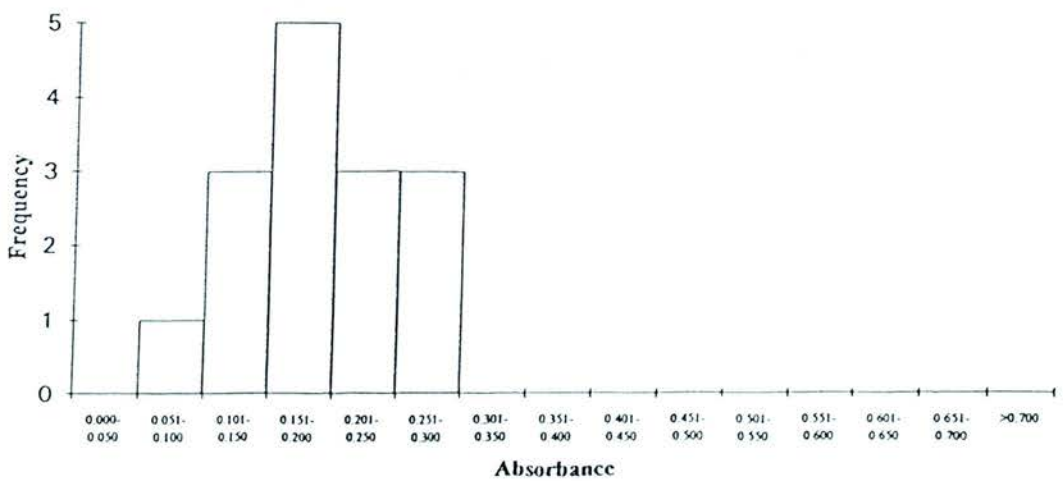
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
>0.700
Absorbance

C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0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
>0.700
Absorbance

8.2.2.2 *B.bigemina* assay

The absorbance values obtained from each duplicate well with the panel of calf serum samples tested against crude *B.bigemina* antigen are presented in Appendix 5.2 and are summarised in Table 8.3. The mean of absorbance values from the anti-*B.bigemina* serum group was approximately 3 times greater than that obtained with pre-infection sera. An anti-*B.bigemina*:anti-*B.bovis* absorbance ratio of 1.8:1 was obtained with the crude *B.bigemina* antigen assay. The greatest variation in absorbance values was observed amongst anti-*B.bigemina* sera (SD = 0.345), followed by the anti-*B.bovis* serum group (SD = 0.201). The pre-infection sera showed the lowest variation (SD = 0.071).

The calculated cut-off point was 0.689. This gave the assay a sensitivity of 0.63, since only 12 amongst 19 known anti-*B.bigemina* sera were detected as positive (Table 8.3). All 15 samples of pre-infection sera were negative. However, as 3 amongst the 13 anti-*B.bovis* sera were detected as positive, 25 amongst 28 samples were detected as true negatives, giving the assay a specificity of 0.89.

The absorbance readings obtained from the anti-*B.bigemina* sera showed that although the majority of the samples had absorbance values higher than 0.900, they were distributed over a wide range of values (Fig 8.2 A). A wide range of values was also observed with anti-*B.bovis* sera (Fig 8.2 B). In contrast, the group of pre-infection sera were distributed in a more limited range (Fig 8.2 C).

Table 8.3 Absorbance values in experimentally raised sera and pre-infection sera tested against crude *B.bigemina* antigen in ELISA.

Sera (No of samples)	Mean	Standard deviation	No of positives/ total
anti-*B.bigemina*(19)	0.848	0.345	12/19
anti-*B.bovis* (13)	0.465	0.201	3/13
pre-infection (15)	0.287	0.071	0/15

Table 8.4 Sensitivity and specificity of ELISA using crude *B.bigemina* antigen.

Status of samples	Proportion
True positives (sensitivity)	0.63 (12/19)
True negatives (specificity)	0.89 (25/28)
False positives	0.11 (3/28)
False negatives	0.37 (7/19)

Fig. 8.2 Frequency distribution of absorbance values obtained with experimentally raised sera and pre-infection sera tested against *B.bigemina* crude antigen in ELISA.

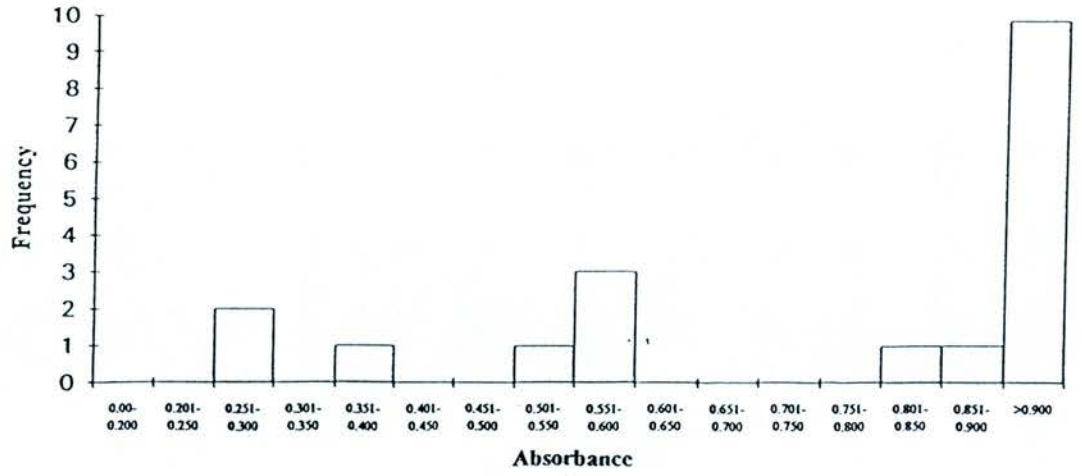
A is anti-*B.bigemina* sera

B is anti-*B.bovis* sera

C is pre-infection sera

Fig 8.2

A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7
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5
4
3
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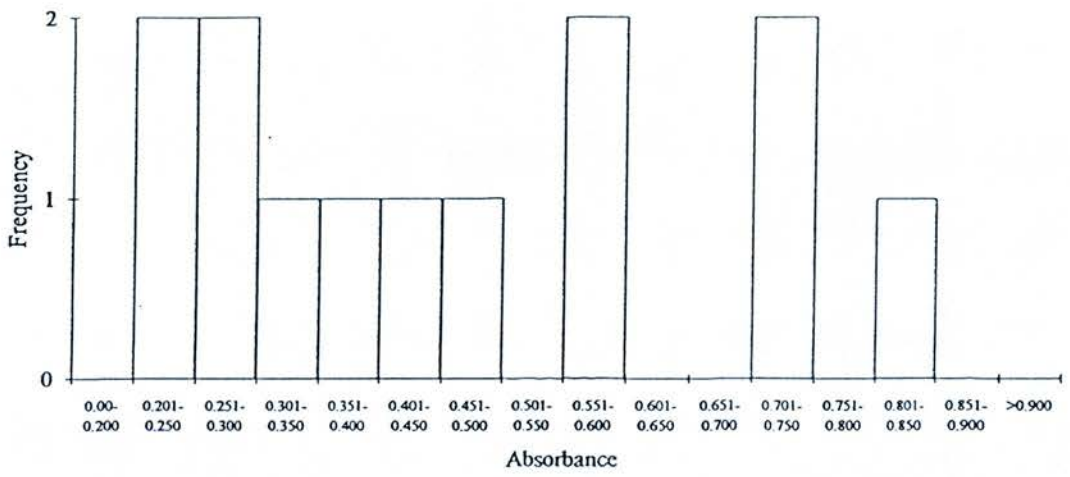
0.201-0.250
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
0.701-0.750
0.751-0.800
0.801-0.850
0.851-0.900
>0.900
Absorbance

B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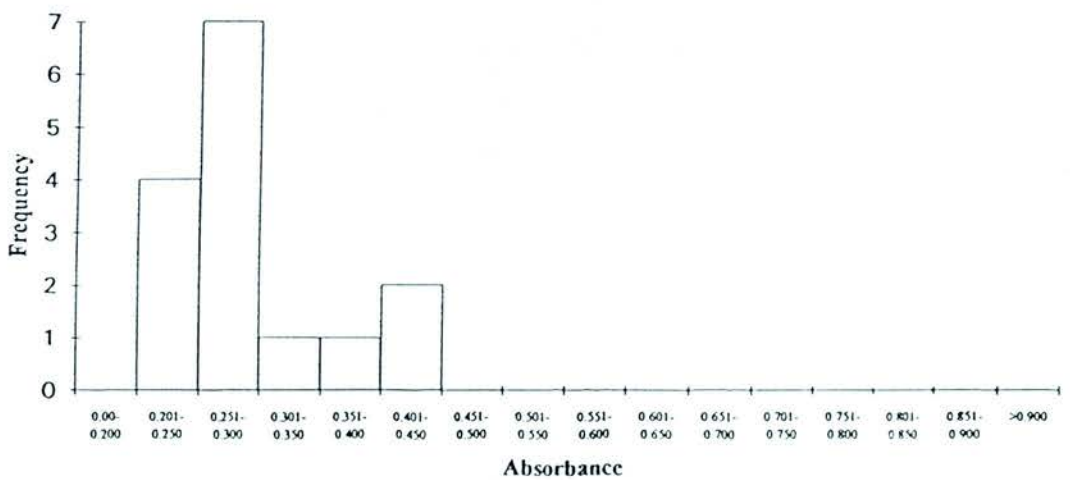
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
0.701-0.750
0.751-0.800
0.801-0.850
0.851-0.900
>0.900
Absorbance

C


Frequency
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0.501-0.550
0.551-0.600
0.601-0.650
0.651-0.700
0.701-0.750
0.751-0.800
0.801-0.850
0.851-0.900
>0.900
Absorbance

8.2.3 Discussion

Discrimination between sera from infected and uninfected animals was achieved with both assays using crude antigens, with mean absorbance values showing homologous positive:negative serum ratios of at least 2.8.

However, cross-reactivity with heterologous sera was observed with both crude antigen preparations. This was more pronounced with the crude *B.bigemina* antigen, since a higher proportion of anti-*B.bovis* sera were detected as positive by the *B.bigemina* antigen than of anti-*B.bigemina* sera detected as positive by the *B.bovis* antigen. Furthermore, the assay using *B.bovis* crude antigen was found to be more sensitive than the assay using *B.bigemina* crude antigen. The observation of two false negative samples with the *B.bovis* crude antigen assay may be related to the fact that the samples had been collected only 17 and 21 days after primary infection, and possibly the level of antibodies was too low to be detected by the assay. Evidence for this hypothesis was given by seroconversion of one of those calves (P78) on day 42 after primary infection. It was not possible to have the same evidence for the other false negative calf (P49), since no sera were collected beyond day 21 after infection.

With the crude *B.bovis* antigen the highest absorbance reading variation between samples (SD) was observed with the anti-*B.bovis* serum group. This was expected since samples had been taken at different times after infection and challenge, and could also be due to individual differences in levels of immune response. For instance, one calf serum (C11) contained a relatively high level of antibodies at 21 days after infection, while two other calves were negative when tested at about the same time after infection. In contrast, low variation in absorbance readings was observed in both anti-*B.bigemina* and pre-infection serum groups, which appeared to be distributed normally. These findings gave support to the decision to calculate a cut-off value based on the mean plus 2 SD.

Defining the positive/negative discrimination level is a crucial element in the design of a serological assay and several methods have been used for this purpose (de Savigny & Voller, 1980). When large numbers (>10) of negative reference sera are available the method most commonly applied is that used in the present study (the mean plus 2 SD). This method assumes that the values are normally distributed and therefore the probability of negative values being below that cut-off point is 95%; however there is a 5% probability of obtaining false positive reactions.

In the present study, the calculated cut-off point value for the *B.bovis* crude antigen assay was considerably higher than that applied by Woodford and co-workers, using the same batch of antigen (Woodford et al, 1990). These authors reported a calculated cut-off point of 0.211, which was also calculated as the mean of absorbance readings from negative reference sera

plus 1.96 times its SD. However, for that calculation Woodford and co-workers used a pool of negative serum samples tested on several occasions (P.Rae, personal communication), whereas in the present study results from mean and SD of individual serum samples from 15 uninfected calves were used to calculate the cut-off point. Obviously, the larger the sample of negative sera, the better approximation it gives of the situation in an unexposed population. Furthermore, the ELISA method used in the present study did not include the step of blocking with 5% normal goat serum in PBS used by Woodford and co-workers. The decision to exclude this blocking stage was based on subsequent work at CTVM which showed that it was not necessary (R.Boid, personal communication). However the absence of that blocking step in the present study may have contributed to increased non-specific background absorbance in the negative serum group, which increased the mean value and consequently resulted in a higher cut-off value than that reported by Woodford and co-workers (Woodford et al, 1990).

The results obtained with the assay using crude *B.bigemina* antigen revealed higher non-specific background readings with negative serum samples than those obtained with the crude *B.bovis* antigen assay. This resulted in a considerably higher cut-off value to enable discrimination between positive and negative samples. However, as the pre-infection serum absorbances were not normally distributed, calculation of the cut-off point by the mean plus 2 SD did not appear to be the most appropriate method for the crude *B.bigemina* antigen assay. Thus, an alternative method for calculation of a cut-off value was attempted based on the use of the mean of the pre-infection serum group multiplied by two. The cut-off point calculated by this method (0.574) would give the assay a sensitivity of 0.74, since 14 amongst 19 anti-*B.bigemina* sera would be detected as positive. Therefore, in order to increase the sensitivity of the crude *B.bigemina* antigen assay, the cut-off point calculated as twice the mean value of negative sera (0.574) was applied in subsequent tests (section 8.4).

However, as several anti-*B.bovis* sera were distributed in a range of absorbances which coincided with some anti-*B.bigemina* sera (as shown in Fig 8.2 A and B), the assay using the *B.bigemina* antigen had a specificity 0.86. These findings indicate that the cross-reactivity between *B.bovis* and *B.bigemina* was more pronounced with the crude *B.bigemina* antigen than with the *B.bovis* antigen. As a consequence, in the present study, neither of the calculated cut-off points for the crude *B.bigemina* antigen assay allowed complete discrimination between anti-*B.bovis* and anti-*B.bigemina* sera. Observation of cross-reactivity higher with *B.bigemina* antigens than with *B.bovis* has also been reported by Wright (1990).

Cross-reactivity between related species of *Babesia* has been demonstrated in other serological tests and very often complicates discrimination of mixed infections in areas where

more than one species is involved (Leeflang & Perie, 1972; Wright et al, 1987; McElwain et al, 1988). Cross-reactivity between *B.bovis* and *B.bigemina* is a particular limitation for epidemiological surveys in endemic areas where both diseases occur. Discrimination between the two infections becomes important for elucidation of particular epidemiological situations and subsequent determination of appropriate methods of control. Cross-reactions between the two species can lead to inaccurate predictions about incidence and prevalence of each disease and, consequently, may result in adoption of inappropriate control measures.

The results from the experiments described in this section point to the need for improvements in specificity of crude *Babesia* antigens, particularly the *B.bigemina* antigen.Therefore the approach of using species-specific *Babesia* proteins to replace crude antigen preparations (described in the next section) was taken with the aim of developing reliable, specific and sensitive tests which would enable discrimination between antibody responses to each species of parasite.

8.3 DEVELOPMENT OF ELISAS USING PROTEINS ELUTED FROM ACRYLAMIDE GELS

8.3.1 Materials and methods

8.3.1.1 Protein elution from gels

Four proteins of *B.bovis* and four of *B.bigemina,* which had been identified as potential candidates for the development of specific ELISAs (as described in Chapters 6 and 7), were selected for elution from acrylamide gels containing parasite antigens. The *B.bovis* proteins had approximate molecular weights of 121, 75, 56 and 46 kDa, and the *B.bigemina* proteins selected for elution had approximate molecular weights of 210, 80, 65 and 50 kDa.

A *B.bovis* lysate (Kwanyanga stock) was prepared from cultured iRBC concentrated by differential hypotonic lysis as described in 3.6.1.

A *B.bigemina* (Mexico stock) lysate was prepared using concentrated cultured iRBC generated after continuous gradient centrifugation with Percoll (as described in Chapter 5).

Crude lysates of *B.bovis* and *B.bigemina* iRBC and reference lanes containing standard molecular weight markers were separated by SDS-PAGE, under reducing conditions, in individual 10% homogeneous gels using the mini-gel system Protean II (Bio-Rad) as described in 3.6.2.

After electrophoresis, the whole gels were stained in Coomassie blue R-250 for 1 hour and destained (as described in section 3.6.3) until the bands were clearly resolved. The bands of interest for each lysate were cut from the gels using a scalpel, and proteins contained in the bands were eluted using the model 422 Electro-Eluter (Bio-Rad). The electroelution was carried out at a constant current of 10 mA/sample for a minimum of 4 hours using electro-elution buffer (Appendix 1). The eluate (approximately 600 µl) recovered from each acrylamide gel slice was then transferred to dialysis tubes (with cut-off level 12 kDa, Sigma) and dialysed against a large volume of ELISA coating buffer (Appendix 1) overnight. The purity and immunogenicity of dialysed proteins were determined by SDS-PAGE (Silver staining) and Western immuno-blotting (as described in sections 3.6.3 and 3.7, respectively) against each respective homologous anti-serum. Eluted proteins were then stored as 100 µl aliquots at -20^{o} C until required for use as antigen in ELISAs.

8.3.1.2 Standardisation of assays

In order to determine the appropriate working dilutions of test sera to be used in ELISAs, the eluted *B.bovis* and *B.bigemina* proteins were tested by ELISA in checkerboard titrations

against an anti-*B.bovis* serum (from calf 396 taken on day 56 after infection), an anti-*B.bigemina* serum (from calf 399 taken on day 57 after infection) and a pre-infection serum (from calf 634 taken on day 0).

Eluted fractions were diluted in coating buffer (Appendix 1) at 1 in 100 dilution and used to coat ELISA plates. Five doubling dilutions (starting from 1 in 100) of each serum sample in PBST were tested in duplicate against each eluted protein. Control wells containing PBS instead of serum were included as a PBS control.

The results from the checkerboard titration were used as the basis for selection of fractions for further development of ELISAs.

The same panel of calf serum samples used for standardisation of crude antigens (as described in 8.2.1.2) were tested in duplicate wells against selected fractions.

The cut-off point for the assay using eluted proteins was calculated as the mean absorbance of the negative sera plus 2 SD.

8.3.2 Results

8.3.2.1 Elution of *B.bovis* proteins

The four *B.bovis* protein bands were successfully eluted from the gel slices, as demonstrated by silver staining of a gel containing samples of each eluted protein (Fig 8.3). Western immuno-blotting analysis showed that the eluted proteins remained antigenic, as demonstrated by detection of reactive bands with molecular weights corresponding to those of the original bands (Fig 8.4). However, after elution, extra antigenic components of proteins 75 and 46 kDa which had not been detected in silver stained gels were recognised by the anti-*B.bovis* serum in immuno-blotting. The protein which showed the strongest reaction in immuno-blotting (56 kDa) was seen to contain a single band.

In ELISA the 56 kDa protein was the only protein which gave *B.bovis*:*B.bigemina* ratios of at least 2.5:1, and absorbance values obtained with anti-*B.bovis* serum 5 times greater than those obtained with the pre-infection serum (results presented in Appendix 5.3). The checkerboard titration indicated that the highest discriminatory ratio between positive and pre-infection sera were obtained with sera diluted at 1 in 200. Titrations of the other three eluted proteins (121, 75 and 46 kDa) did not result in discrimination between pre and post-infection sera (see Appendix 5.3). Therefore, the 56 kDa protein was selected to be used as antigen for the development of a *B.bovis*-specific antibody detection ELISA.

In the subsequent tests, the 56 kDa eluted protein was used to coat plates at a dilution of 1 in 100 and test sera were diluted at 1 in 200.

8.3.2.2 ELISA using the eluted *B.bovis* 56 kDa protein

The absorbance values obtained from each duplicate well with the 56 kDa *B.bovis* protein tested against the panel of calf sera are presented in Appendix 5.5 and are summarised in Table 8.5. Positive:negative as well as *B.bovis*:*B.bigemina* absorbance ratios of only 1.5:1 were obtained with the assay using the 56 kDa protein as antigen. The greatest variation in absorbance values was observed amongst anti-*B.bovis* sera (SD = 0.132), whereas anti-*B.bigemina* and pre-infection sera showed little variation (SD = 0.059 and 0.049, respectively).

The calculated cut-off point was 0.352, which gave the assay a sensitivity of 0.61, since only 8 of 13 known anti-*B.bovis* sera were detected as positive (Tables 8.5 and 8.6). All 15 samples of pre-infection sera were negative. The specificity of this assay was 0.94, since two anti-*B.bigemina* serum were detected as positive.

The absorbance readings obtained from the anti-*B.bovis* sera showed that the majority were distributed within a wide range of absorbance (Fig 8.5 A). In contrast, the anti-*B.bigemina* and pre-infection sera were distributed with in a more limited range of values, resembling a normal distribution (Fig 8.5 B and C).

Fig 8.3 Silver staining of a 10% homogeneous gel containing *B.bovis* eluted proteins from gel slices.

Lane 1 contains the crude lysate of *B.bovis*, lane 2 contains the 121 kDa band, lane 3 contains the 75 kDa band, lane 4 contains the 56 kDa band and lane 5 contains the 46 kDa band.

Fig 8.3

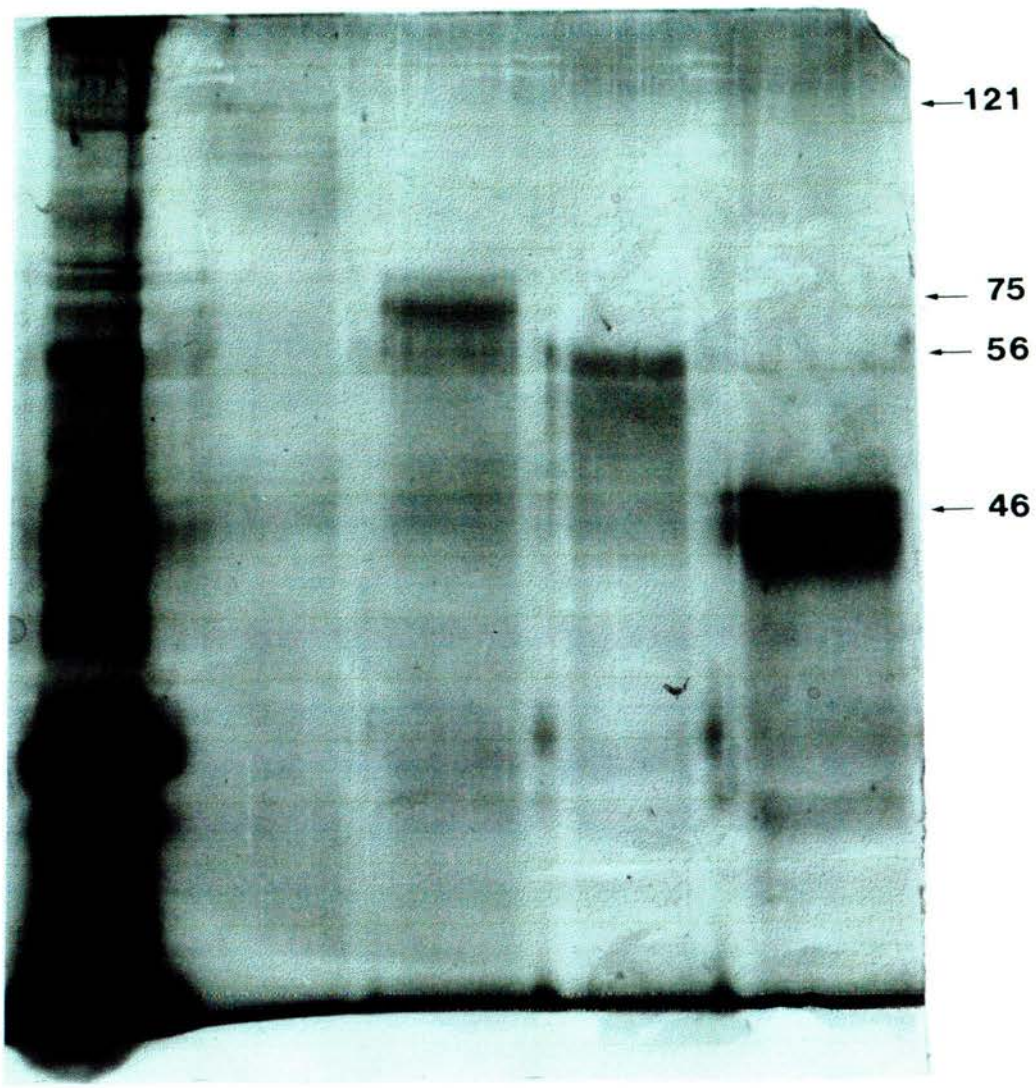


Fig 8.4 Western immuno-blotting of *B.bovis* eluted protein bands against anti-*B.bovis* serum. Lane 1 contains the crude lysate of *B.bovis*, lane 2 contains the 46 kDa band, lane 3 contains the 56 kDa band, lane 4 contains the 75 kDa band and lane 5 contains the 121 kDa band.

Fig 8.4

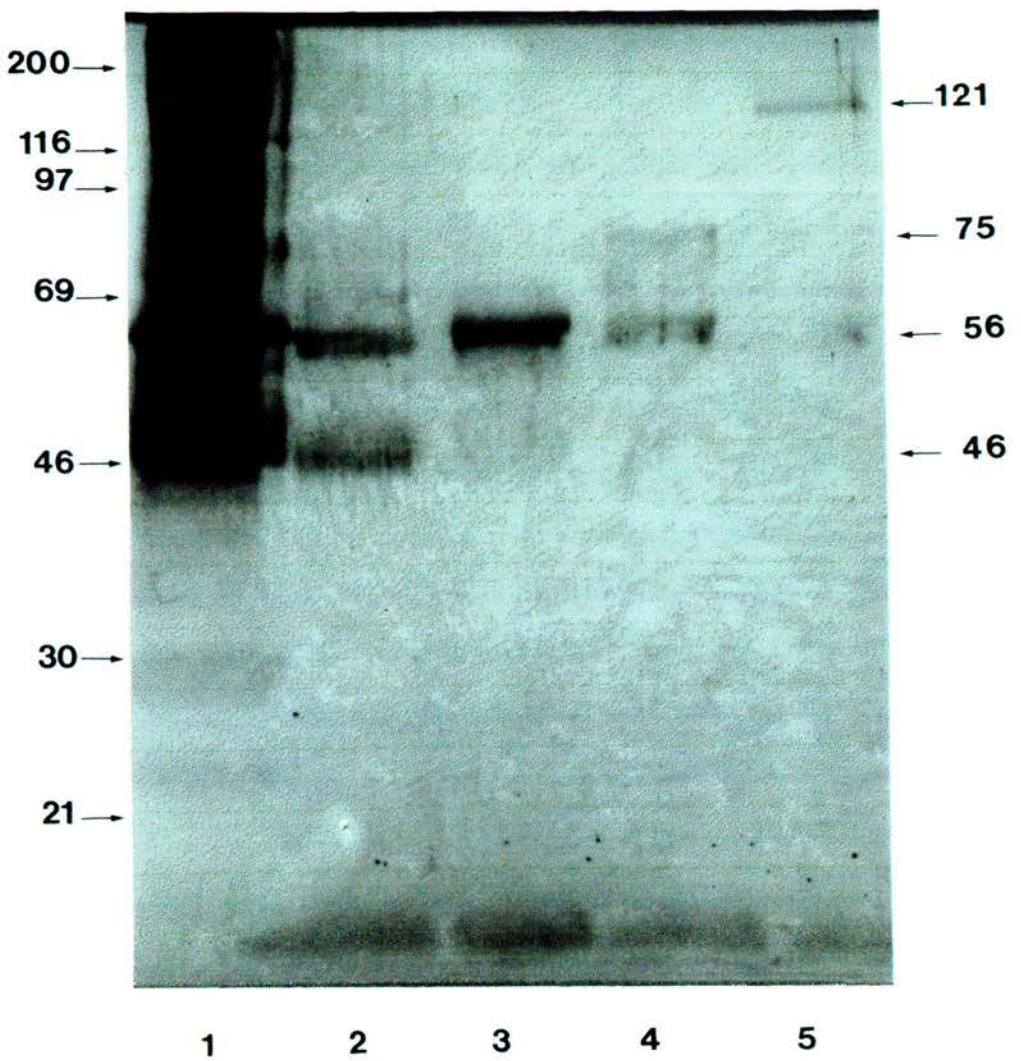


Table 8.5 Absorbance values in experimentally raised sera and pre-infection sera tested against *B.bovis* 56 kDa protein in ELISA.

Sera (No of samples)	Mean	Standard deviation	Number of positives/ total
anti-*B.bovis* (13)	0.372	0.132	8/13
anti-*B.bigemina*(19)	0.275	0.059	2/19
pre-infection (15)	0.254	0.049	0/15

Table 8.6 Sensitivity and specificity of ELISA using an eluted *B.bovis* 56 kDa protein as antigen.

Status of samples	Proportion
True positives (sensitivity)	0.61 (8/13)
True negatives (specificity)	0.94 (32/34)
False positives	0.05 (2/34)
False negatives	0.38 (5/13)

Fig 8.5 Frequency distribution of absorbance values obtained with experimentally raised sera and pre-infection sera tested against *B.bovis* 56 kDa protein.

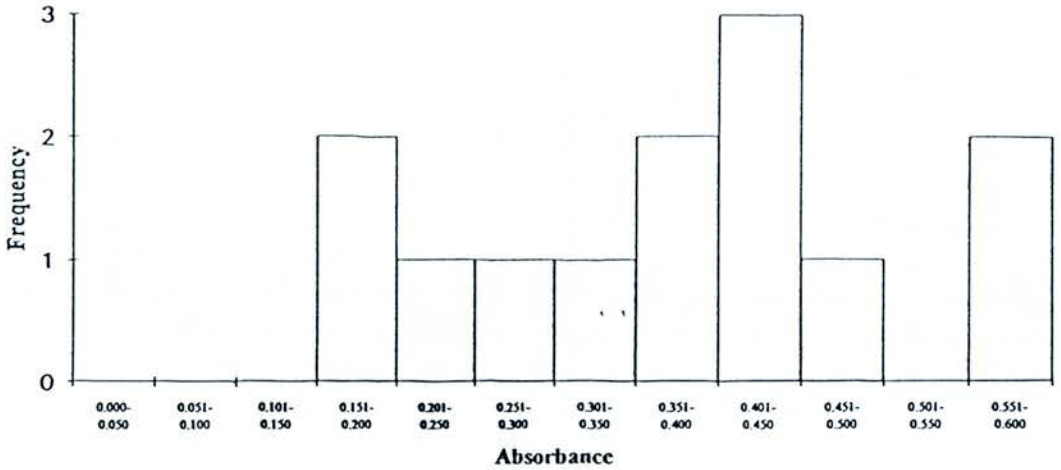
A is anti-*B.bovis* sera

B is anti-*B.bigemina* sera

C is pre-infection sera

Fig 8.5

A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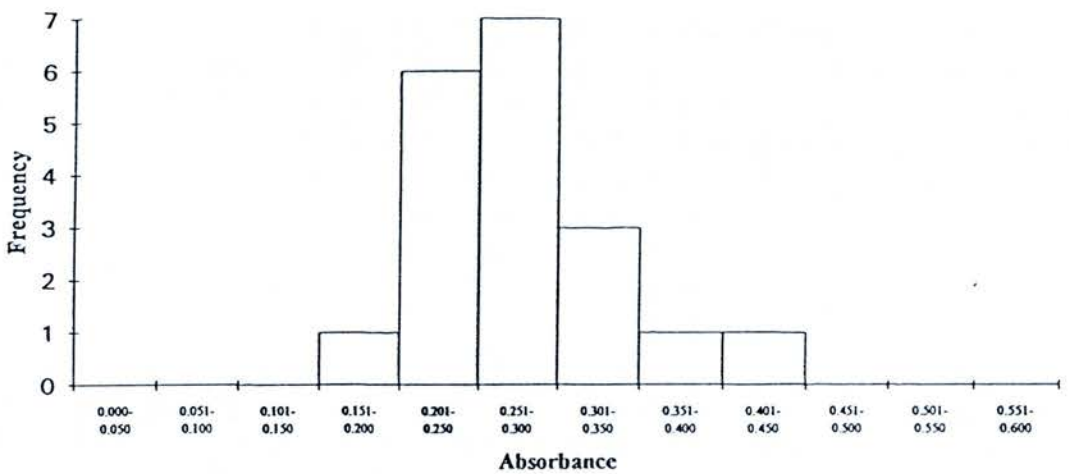
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
Absorbance

B


Frequency
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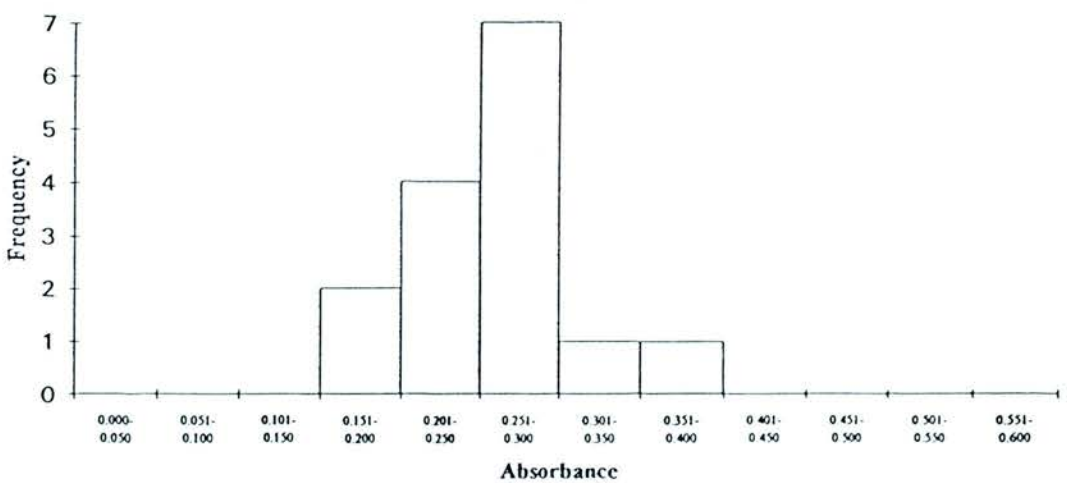
0.201-0.250
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
Absorbance

C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2
3
4
5
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0.201-0.250
0.251-0.300
0.301-0.350
0.351-0.400
0.401-0.450
0.451-0.500
0.501-0.550
0.551-0.600
Absorbance

8.3.2.3 Elution of *B.bigemina* proteins

The four target proteins were successfully eluted from gel slices, as demonstrated by silver staining of a gel containing samples of each eluted protein (Fig 8.6.). However, Western immuno-blotting analysis of the eluted proteins showed that only two proteins (65 and 50 kDa) remained antigenic after elution, demonstrated by detection of reactive bands with molecular weights corresponding to those of the original bands (Fig 8.7). The reaction detected against the 65 kDa was weaker than that detected against the 50 kDa protein.

However, ELISA checkerboard titrations showed no discrimination between homologous, heterologous and pre-infection sera with any of the eluted proteins (results presented in Appendix 5.4).

Therefore, it was not possible to develop a *B.bigemina* specific-ELISA test using the proteins eluted from acrylamide gels.

Fig 8.6 Silver staining of a 10% homogeneous gel containing *B.bigemina* eluted proteins from gel slices.

Lane 1 contains the 50 kDa band, lane 2 contains the 65 kDa band, lane 3 contains the 80 kDa band and lane 4 contains the 210 kDa.

Fig 8.6

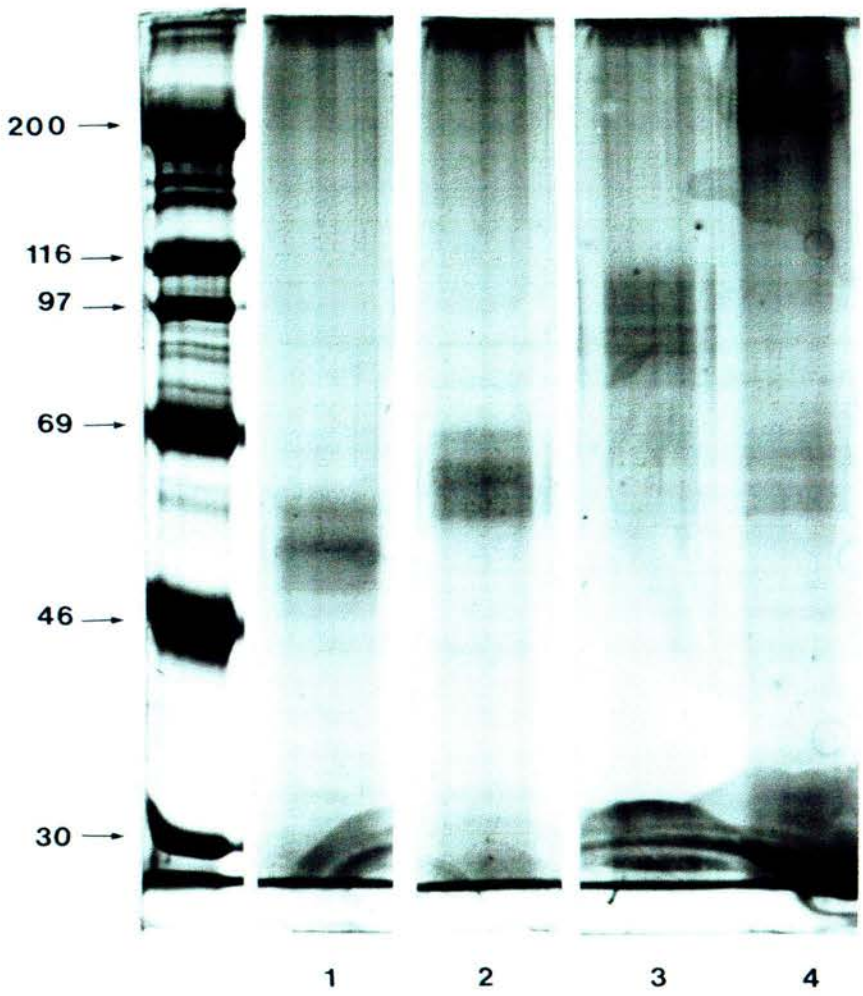
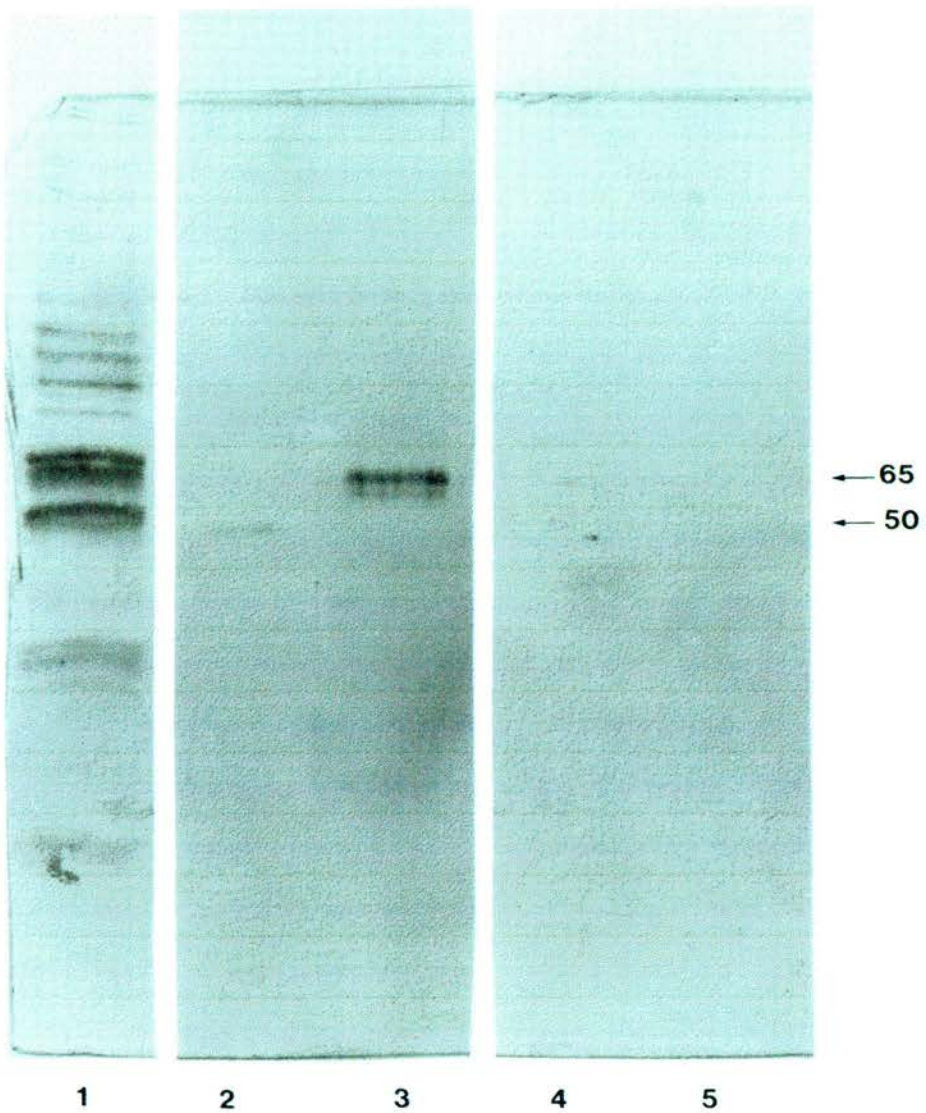


Fig 8.7 Western immuno-blotting of *B.bigemina* eluted proteins against anti-*B.bigemina* serum.

Lane 1 contains the crude lysate of *B.bigemina*, lane 2 contains the 50 kDa band, lane 3 contains the 65 kDa band, lane 4 contains the 80 kDa band and lane 5 contains the 210 kDa band.

Fig 8.7



8.3.3 Discussion

Purification of parasite components was successfully achieved by electroelution of particular protein bands from crude preparations, as demonstrated by SDS-PAGE analysis (Fig 8.3). Silver staining was used to determine the purity of eluted proteins due to its high sensitivity in detecting small amounts of proteins. Silver staining is up to 100 times more sensitive than Coomassie blue, and is able to detect as little as 0.38 ng/mm^2 of bovine serum albumin (Hames & Rickwood, 1990). Although single bands were seen in each of the eluted fractions of *B.bovis*, Western immuno-blotting analysis (Fig 8.4) showed the presence of more than one band in two fractions (75 and 46 kDa). This might be due to cleavage of the protein into smaller molecular weight units as a result of the reducing effect of the sample buffer. Only one fraction of *B.bovis* (56 kDa) showed a single band by both silver staining and Western immuno-blotting.

Results from ELISA checkerboard titrations showed that only the 56 kDa eluted protein resulted in acceptable discrimination between anti-*B.bovis*, anti-*B.bigemina* and pre-infection sera, with *B.bovis*:*B.bigemina* absorbance ratios of at least 2.5:1, and *B.bovis*:negative absorbance ratios of 5:1. None of the other eluted proteins showed discrimination between pre and post-infection sera. Results from the titrations indicated that the three *B.bovis* fractions (121, 75 and 46 kDa) reacted equally against anti-*Babesia* sera and uninfected serum and therefore the reactions were non-specific. This may be due to a loss of antigenicity of *B.bovis* specific epitopes during the extensive purification procedure, which involved denaturing of the proteins by the addition of sample buffer and electroelution over a relatively long period of time (minimum of 4 hours). Thus, although antigenic bands were detected in Western immuno-blotting, the antigenicity of these three *B.bovis* components was not preserved in the ELISA. Furthermore, it is also possible that, after the various steps of the purification procedure, the specific epitopes had lost their ability to bind to ELISA plates, despite the fact that the eluted fractions had been dialysed against coating buffer before being used in ELISAs.

Purification of *B.bigemina* protein components was also successfully achieved by electroelution, as demonstrated by silver staining of acrylamide gels (Fig 8.6). However the Western immuno-blotting analysis demonstrated that only two fractions (65 and 50 kDa) remained antigenic, with the strongest reaction being detected against the 50 kDa fraction. The absence of detectable immuno-reaction against the other fractions may have been related to either low protein concentration in these fractions, since their protein bands were weakly detected in silver staining, or loss of antigenicity during the elution procedure. Results from ELISAs showed discrimination between pre and post-infection sera but not between anti-*B.bovis* and anti-*B.bigemina* sera. This was seen with all of the fractions, suggesting that all

four proteins may have epitopes shared by the two parasites and thus none of them was *B.bigemina* specific. These findings reinforce the theory that *B.bigemina* has more epitopes cross-reactive with *B.bovis* than vice-versa, as indicated by the ELISAs carried out using crude parasite preparations (section 8.2).

As the 56 kDa *B.bovis* protein was the only one that remained antigenic in ELISA after the purification procedure, and showed good discrimination between anti-*B.bovis* and anti-*B.bigemina* sera, it was tested against the panel of known sera. Results from these ELISAs showed lower mean absorbance ratios of both anti-*B.bovis*:negative sera (1.5:1) and anti-*B.bovis*:anti-*B.bigemina* sera (1.5:1) than those obtained with single samples of each type of serum in the checkerboard titrations, which showed 5:1 and 2.5:1, respectively. High background readings were obtained for the negative serum group, in contrast to low absorbance values obtained from the pre-infection serum in the checkerboard titration.

Thus, applying the calculated cut-off value to determine the status of known calf sera resulted in a sensitivity of 0.61 and a specificity of 0.94 for the assay using the *B.bovis* 56 kDa protein. The sensitivity of the assay was lower than expected, with a large proportion of infected calves (5/13) not being detected as positives. However, these false negative samples had been collected relatively early after primary infection (on average 27 days p.i.). It is possible that the immune response against this particular *B.bovis* component (56 kDa) develops later after infection and that sera from early stages of infection did not contain antibodies against it. This could be due to, for instance, a small proportion of the 56 kDa component in relation to other *B.bovis* immunogenic components being responsible for induction of immune responses at an early stage after primary infection. Therefore, the 56 kDa might be useful to differentiate between chronic and early infections. Furthermore, two anti-*B.bovis* samples which were falsely negative in the assay using the 56 kDa protein, were also falsely negative in the assay using crude *B.bovis* antigen. This suggested that inherent characteristics of these calves, such as reduced responsiveness to *Babesia* infections, or development of immune response later than in the other calves, could have been responsible for the lack of detection of anti-*Babesia* specific antibodies in these samples.

The two false positive samples in the assay using the *B.bovis* 56 kDa protein were from a single calf (397), taken at different stages after *B.bigemina* infection. This may be related to an immune response developed in this animal against other unrelated antigens that may have similar epitopes to that of the 56 kDa protein of *B.bovis*. It appeared that the reactions were due to an individual characteristic of that calf rather than to cross-reactivity between *B.bovis* and *B.bigemina*, since none of the sera from the other calves infected with *B.bigemina* cross-reacted with the *B.bovis* 56 kDa protein.

Overall, one has to consider that sera produced experimentally against *Babesia* parasites, such as the ones used in the present study, very often do not represent the reality observed in the field, and therefore are not the ideal reference sera. There are several factors related to the initiation and subsequent course of a babesial infection that certainly will affect the development of the immune response. The most relevant is probably related to the source and nature of parasite immunogens. For the calf immunisations carried out in the present study, almost all *Babesia* material used was blood-derived. The immunisation of calves was achieved using as inoculum cryopreserved *Babesia*-infected RBC, either derived from infected blood or from culture suspensions originating from infected blood. This immunisation scheme is quite different from what happens under natural field conditions, where the animals are infected through inoculation by infected ticks of infective sporozoite stages of *Babesia*. The use of infected ticks for immunisation of calves was not possible at CTVM, since this would involve maintainance of *Boophilus* tick colonies with pure *Babesia* infections (both the tick and *Babesia* being species exotic to Britain) over a long period of time, and a large number of calves would be necessary. In the present study, a *B.bigemina* calf infection was carried out by infestation with infected ticks on only one occasion, when *B.decoloratus* larvae had been brought from Kenya. However, that infected calf was destroyed during ascending parasitaemia (day 23 p.i.), before the development of specific antibodies. In all the other immunised calves, the immune response must have been directed against blood stages of the parasite (trophozoites and merozoites), which were the only forms present in the inoculum. The development of differential cellular immune responses to *B.bovis* according to the source of inoculum used (tick-derived parasites or cultured merozoites) has been reported recently (Brown et al, 1991). Thus, it is possible that development of differential humoral immune responses according to the type of inoculum used also occurs in *Babesia* infections.

Another difference between the artificial infections of the present study and natural infections in the field is that in endemic areas the animals are under constant natural tick challenge, which may contribute to the development of stronger immune responses than those produced artificially by needle inoculations at particular intervals.

Furthermore, under natural field conditions the animals are usually exposed to a variety of other agents, which stimulate the immune system and may interact with immunogens of babesial origin. Although the animals used in the present study were not kept isolated from other agents, they were exposed to a much more limited range of agents (both pathogens and non-pathogens) than animals in tropical *Babesia*-endemic areas, where a large number of organisms are present in the environment, and capable of stimulating the immune system.

With all these considerations in mind, the *B.bovis* 56 kDa protein was considered to be species-specific and a further experiment was designed to validate its use as a *B.bovis* specific antigen in an antibody detection ELISA for use in *Babesia* endemic areas.

8.4 COMPARATIVE ANALYSIS OF ELISAS USING CRUDE ANTIGENS AND ELUTED PROTEINS

8.4.1 Materials and methods

For this analysis a panel of serum samples collected from cattle exposed to natural tick infestation under field conditions (as described in section 3.4.2) was tested against both *B.bovis* and *B.bigemina* crude antigens, and the *B.bovis* 56 kDa eluted protein. The panel included 33 samples collected in Mozambique, 49 samples collected in Malawi and 7 samples collected in Brazil.

Both crude antigens were tested at a dilution of 1 in 1,000 with test sera diluted at 1 in 100, whereas the *B.bovis* 56 kDa eluted protein was tested at a dilution of 1 in 100 with test sera diluted 1 in 200. In both assays (crude antigen and 56 kDa protein) anti-bovine IgG peroxidase conjugate was used at a dilution of 1 in 6,000; all test serum samples were tested in duplicate wells. A serum sample was considered positive if its mean calculated from the duplicate wells was greater than the respective cut-off points for each assay.

In order to minimise plate-to-plate variations between assays performed at different times, the means of absorbance values were adjusted using a factor which was calculated from readings of control wells of each plate, as described below:

$$\text{Factor (F)} = \frac{Po - No}{Pt - Nt}$$

$$\text{Adjusted value} = F(St - Nt) + No$$

Po = Mean of 4 positive control wells in first plate

No = Mean of 4 negative control wells in first plate

Pt = Mean of 4 positive control wells in subsequent test plate

Nt = Mean of 4 negative control wells in subsequent test plate

St = Mean of 2 wells of test sample

Based on the standardisation of each assay, the values considered positive were absorbance values > 0.310 for the assay using crude *B.bovis* antigen, > 0.352 for the assay using the *B.bovis* 56 kDa protein, and > 0.574 for the assay using crude *B.bigemina* antigen.

The two assays for detection of anti-*B.bovis* antibodies (using crude antigen or the 56 kDa protein) were compared using correlation analysis (Fowler & Cohen, 1990) considering the mean absorbance readings obtained from each field serum sample in each assay.

8.4.2 Results

8.4.2.1 *B.bovis* assays

The absorbance values from each duplicate well and the means and adjusted values obtained from the panels of field serum samples tested against the crude *B.bovis* antigen and against the 56 kDa protein are presented in Appendix 5.6 and 5.8 respectively, and are summarised in Table 8.7.

All 33 samples from Mozambique and all 7 from Brazil were positive by both assays (crude antigen and the 56 kDa protein), resulting in agreement of 100% between the two tests. The agreement between the two tests was 95.6% in relation to the Malawi sera. Amongst the 5 samples detected as negative by the crude antigen assay, 3 were also negative in the assay using the 56 kDa protein. However the other two samples detected as negative in the crude antigen assay were detected as positive in the assay using the 56 kDa protein. The disagreement of 4.4% between the two tests was not significant when the results were compared by the Chi-square test.

The analysis of correlation between the mean absorbances obtained for each sample in each assay resulted in a calculated correlation coefficient of 0.44, which for the number of observations (89) means that there is modest positive linear correlation between the results from the two tests. This is represented in Fig 8.8.

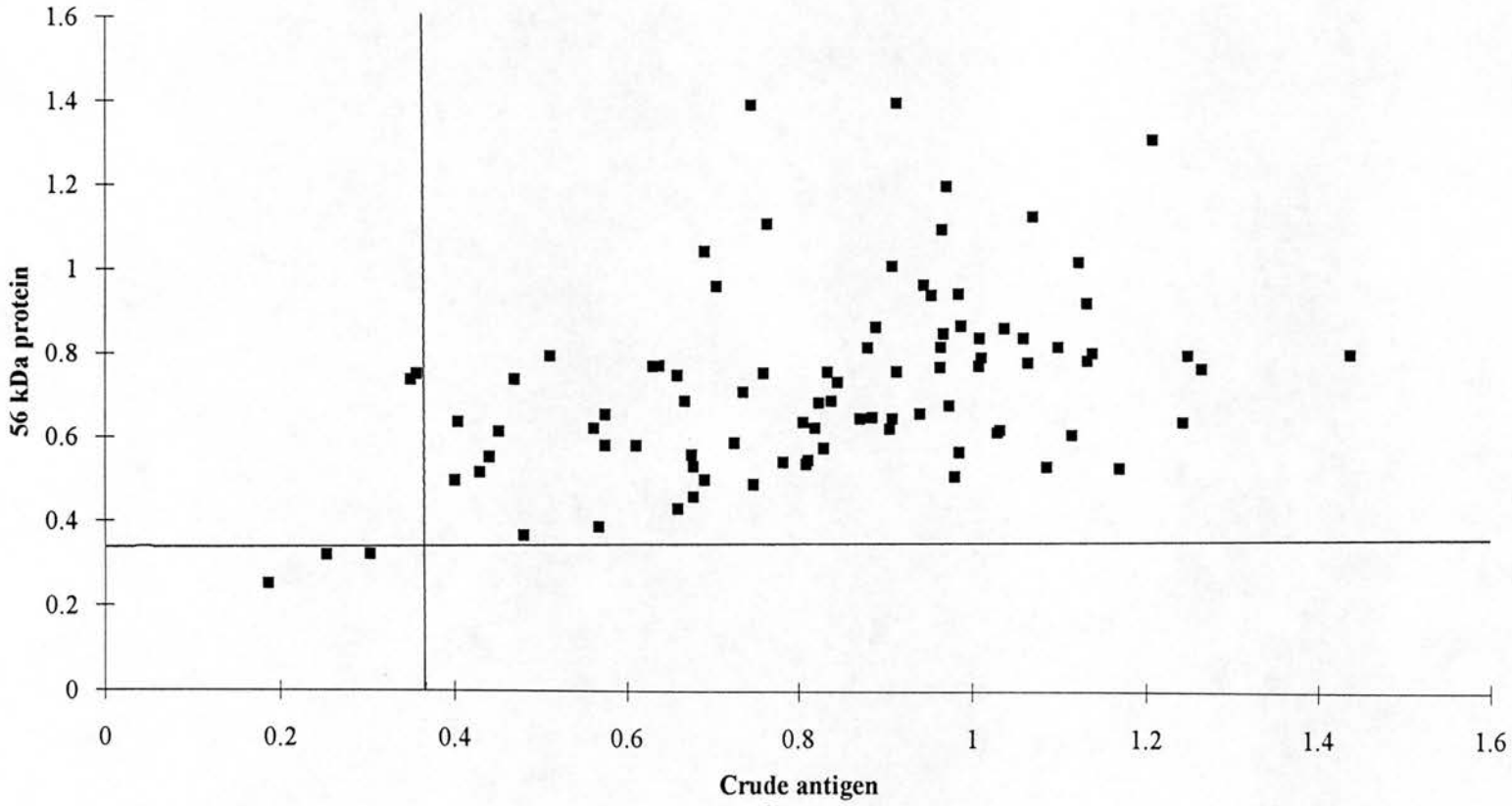
Table 8.7 Number of positive field serum samples in ELISAs using either crude *B.bovis* antigen or the 56 kDa eluted protein as antigen.

Sample origin	Crude antigen		56 kDa protein		Agreement (%)
	+ve	-ve	+ve	-ve	
Mozambique	33	0	33	0	100.0
Malawi	44	5	46	3	95.6
Brazil	7	0	7	0	100.0

Key: +ve = positive
-ve = negative

Fig 8.8 ELISA absorbance values obtained with field sera tested against both crude *B.bovis* antigen and the 56 kDa protein.
Overlying lines represent the calculated cut-off points for each assay.

Fig 8.8


56 kDa protein
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Crude antigen

8.4.2.2 Crude *B.bigemina* antigen assay

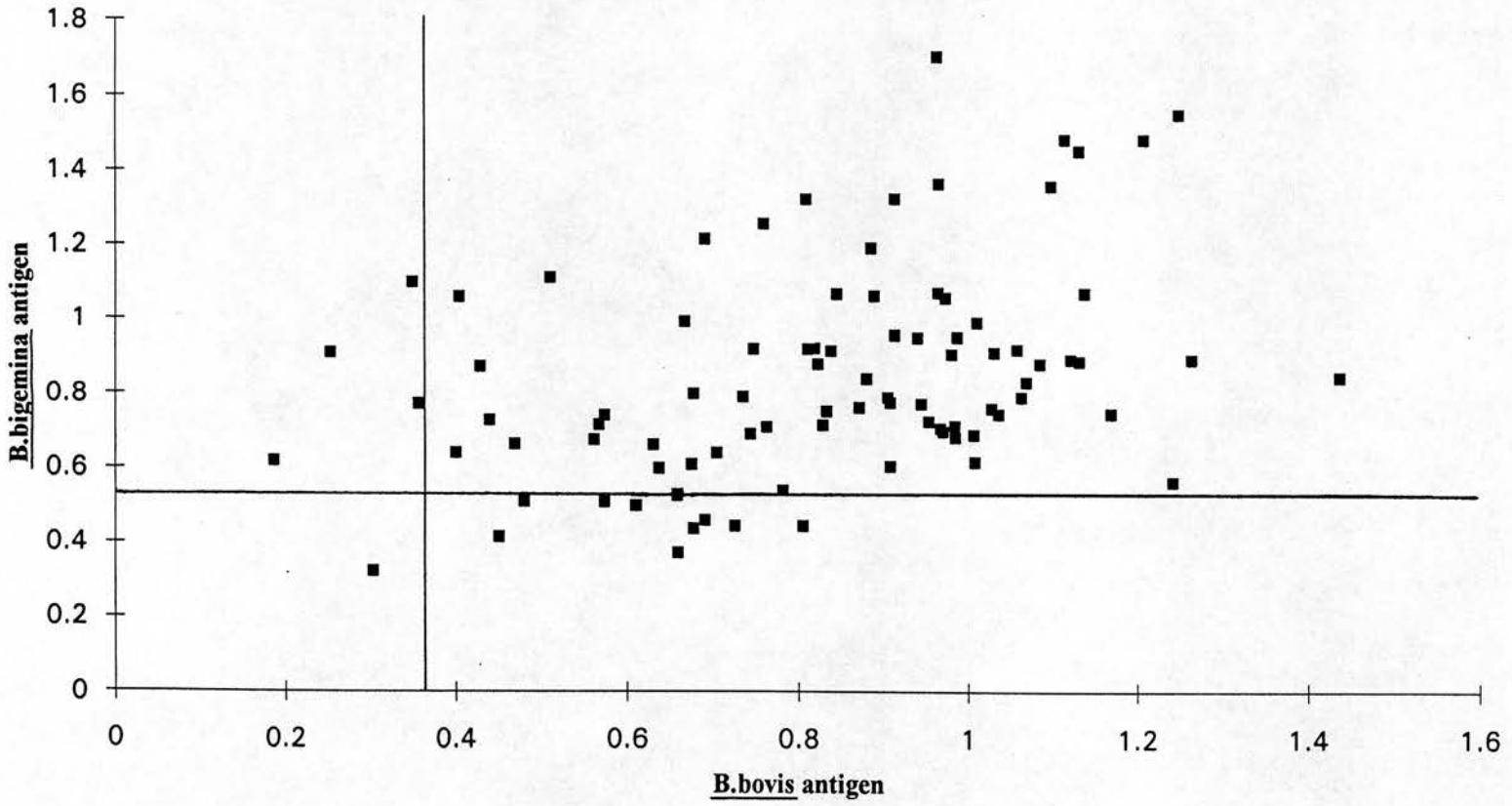
The assay using the crude *B.bigemina* antigen detected as positive 25 (78.8%) serum samples from Mozambique out of 33 tested, 47 (95.9%) out of 49 serum samples from Malawi, and 6 (85.7%) out of 7 samples from Brazil. The absorbance values of each pair of wells, the means and the adjusted values obtained from field serum samples against crude *B.bigemina* antigen are presented in Appendix 5.7. Only one serum sample, from Malawi (L243), was detected as negative by all of the tests (*B.bovis* and *B.bigemina* crude antigens and the 56 kDa purified protein).

The mean absorbances obtained from each field serum sample with both crude antigen assays (*B.bovis* and *B.bigemina*) were compared and the calculated correlation coefficient was 0.37, which for the number of observations (89), indicates that there is a moderate positive linear correlation between results obtained in the two tests.

Fig 8.9 summarises the results obtained with both crude antigens (*B.bovis* and *B.bigemina*) and shows that the vast majority of the field samples tested were detected as positive by both assays; however amongst the samples detected as negative, a larger number were negative with the *B.bigemina* antigen than with *B.bovis*.

Fig 8.9 ELISA absorbance values obtained with field sera tested against crude antigens of *B.bovis* and *B.bigemina*.
Overlying lines represent the calculated cut-off points for each assay.

Fig 8.9


1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
B.bigemina antigen
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
B.bovis antigen

8.5 GENERAL DISCUSSION AND CONCLUSIONS

The antibody detection ELISA developed using the *B.bovis* 56 kDa antigen gave results comparable to that obtained with the assay using the reference crude antigen.

The standardisation of the assay using the panel of known calf sera resulted in relatively low sensitivity (0.61) in relation to that achieved with the crude antigen assay (0.85). However, the validation of the developed test using a panel of bovine field sera originating from three different countries proved that the purified 56 kDa protein was as good as the crude parasite preparation in detecting reactive sera and that it could replace the latter in sero-epidemiological studies in endemic areas. Although the quantitative correlation between absorbance values obtained by each assay was low, an overall qualitative agreement of 97.8% was observed between the assays.

The vast majority of the field sera tested were positive to both of the crude antigens and the 56 kDa *B.bovis* protein, with only one animal being detected as negative to all three antigenic preparations. This was not surprising as they originated from endemic areas, where a high inoculation rate of *Babesia* is expected under natural conditions, and consequently a high proportion of the animals should produce specific antibodies.

Considering that the field sera tested here had been taken from animals older than 12 months, and therefore probably chronic carriers, the results obtained with the 56 kDa antigen assay give support to indications that it may be useful in detecting chronic infections. Although the assay using the 56 kDa antigen was shown to be less sensitive than the crude antigen assay during standardisation (see Tables 8.2 and 8.6), it detected a higher number of positive field samples than did the crude antigen assay, and indicated that this antigen was conserved between strains. Detection of a higher number of positive samples by the 56 kDa antigen was not expected, since crude antigenic preparations, which contain numerous parasite antigens, would be expected to detect at least as many positive samples as a pure antigen. Only two samples (2.2% of the total tested) that were detected as negative by the crude antigen were positive by the 56 kDa antigen assay; these can be regarded as within the expected percentage of false negatives with the crude antigen assay (15%). Thus, although the panel of known experimental sera indicated low sensitivity (0.61) for the 56 kDa antigen assay, a higher number of positive field sera were detected using this test. This may be due to the presence of higher titres in sera collected from cattle naturally exposed to *Babesia* infections in the field than in experimentally infected animals, since in natural field conditions the animals are under constant challenge.

In addition, as reported by Jacobson (1992), when the disease in question has a high expected prevalence, as in the case of *Babesia* infections in endemic areas, negative results are usually

good indicators of non-infected animals even if the sensitivity and specificity of the test are not very high. However, in situations were prevalence is low, a test with a sensitivity of 99% and a specificity of 99% becomes a poor predictor of infection. In this situation with a disease prevalence of 0.1%, a positive result would be wrong 91% of the time (Jacobson, 1992).

A purified parasite specific antigen would be preferable to crude antigenic preparations for sero-diagnostic tests in many ways. Common limitations related to the use of crude preparations, which it is hoped to overcome with the use of species-specific components, are the cross-reactivity between related species and non-specific reactions with host contaminants. Another limitation of crude antigens is the potentially large batch-to-batch variation, which relates to the parasitaemia of infected blood or culture suspension used, amongst other factors such as efficiency of concentration and purification procedures. This limitation could also be overcome with the use of pure parasite fractions, which would be produced reproducibly either as a recombinant fusion protein by recombinant DNA technology or by artificial synthesis once its amino acid composition had been sequenced. Furthermore, the use of synthetic antigens would eliminate the need to sacrifice experimental cattle for the preparation of crude antigens.

Since the first use of the ELISA system for detection of antibodies against *B.bovis* (Barry et al, 1982) efforts have been made to improve the quality of crude antigenic preparations, in order to increase sensitivity and specificity of assays. Recently, Bose and co-workers reported for the first time an ELISA test using a recombinant *B.bovis* antigen (11C5) expressed in *Escherichia coli*, which was shown to be both sensitive and specific as compared with a native antigen preparation (Bose et al, 1990). Later, the same 11C5 recombinant antigen was found to have a high degree of cross-reactivity with *B.ovis* and, as a result, the ELISA developed for detection of anti-*B.bovis* antibodies was found to be useful for immunodiagnosis of *B.ovis* (Duzgun et al, 1991). The native antigen 11C5 was identified by a monoclonal antibody that reacted against a large number of closely positioned bands in Western immuno-blotting (Goodger et al, 1992a) and therefore it appears to be distinct from the *B.bovis* 56 kDa used in the present study. As described here for the 56 kDa antigen, the 11C5 recombinant antigen showed lower sensitivity when compared with a crude antigen preparation in ELISAs using known status sera, and there was a suggestion that the use of a cocktail of two or more purified antigens could overcome the possible low sensitivity of a single antigen (Bose et al, 1990). Thus, the 56 kDa antigen used in the present study constitutes an appropriate candidate for further investigation in association with other well defined antigens.

The high background absorbance readings, which are commonly observed with crude antigenic preparations due to the presence of contaminants, were also observed with the 56

kDa antigen assay. This was not expected from a purified antigen. It may be regarded as a consequence of the extensive purification procedure, which may have contributed to a loss of the specificity of the antigen. However, even with the non-specific background reading it was possible to discriminate between positive/negative sera, and this did not appear to interfere with the interpretation of results from the field sera. Nevertheless, a decrease of the non-specific background is desirable and might be achieved with further purification of the 56 kDa antigen by conventional methods such as precipitation, immuno-affinity and dialysis.

Unfortunately it was not possible to develop a *B.bigemina*-specific ELISA using eluted proteins. This would be useful for discrimination between *B.bovis* and *B.bigemina* infections in areas endemic for both parasites. The need for the development of a *B.bigemina*-specific ELISA was evident from the poorer performance of the *B.bigemina* crude antigen in comparison to that of *B.bovis*. This was inferred by higher cross-reactivity and non-specific background obtained with the crude antigenic preparation of *B.bigemina* than with the *B.bovis* crude antigen. The lack of an assay with a *B.bigemina* purified antigen limited the validation of the *B.bovis* 56 kDa using field serum samples, in relation to determination of cross-reactivity.

As observed with both *B.bovis* antigens, the majority of the field sera were positive when tested against the *B.bigemina* crude antigen. However the proportion of negative animals was slightly higher than that obtained with *B.bovis* antigens. This was not expected, since in an endemic situation where the two infections occur together, inoculation rates of *B.bigemina* are usually higher than those of *B.bovis* (Miller et al, 1984). This is due to the particular nature of each parasite's life cycle in the vector tick. *B.bovis* is transmitted only by the larval stage which then loses the infection and vertical transmission does not occur (Mahoney & Mirre, 1979). The infection of the tick population with *B.bovis* is therefore based only on alimentary infection of the engorging females which will produce a low proportion of infected eggs. *B.bigemina*, on the other hand, is transmitted by feeding nymphs, females and males, and in addition to the alimentary infection, there is also vertical transmission which results in maintenance of the infection over two or more generations of the tick without alimentary reinfection (Friedhoff, 1988). These particular characteristics result in higher rates of *B.bigemina* infection in ticks. However, the slightly higher prevalence of anti-*B.bovis* antibodies may have been observed because the number of samples tested was small and probably do not fully represent the epidemiological situation in each area where the samples originated.

In conclusion, the ELISA developed for detection of antibodies against *B.bovis* using a purified 56 kDa antigen was shown to be appropriate for use in epidemiological studies.

Further studies on this antigen regarding its composition, origin and dynamics during *B.bovis* infections should be carried out with the aim of producing it synthetically for large scale use.

Some of the work reported in this chapter has been published (see Appendix 6.3):

Passos, L.M.F.; Brown, C.G.D.; Jones,T.W.; Bell-Sakyi, L.; Boid, R. (1993) The development of specific ELISAs for use in epidemiological studies of bovine babesiosis in Brazil. *Proceedings of the 14th International Conference of the World Association for the Advancement of Veterinary Parasitology*, Cambridge.

CHAPTER NINE

CONCLUSIONS

The aim of the present study was to develop ELISAs using purified proteins for detection of specific antibodies against *B.bovis* and *B.bigemina*, with which to discriminate between the two infections in endemic areas. Such discrimination would contribute to more effective targeting of control measures against bovine babesiosis. The approach used here for identification of potential candidate proteins of each species was based on immunochemical characterisation of different stocks of these *Babesia* species.

Two constraints were faced in the present study on immunochemical characterisation of *Babesia* stocks. The first was related to the provision of parasites on a regular basis, for which *in vitro* culture systems were considered to be the most appropriate source of material to be used at CTVM, and the second was related to the low parasitaemias usually obtained in both infected blood and culture suspensions, from which concentration of either parasites or iRBC was necessary in order to obtain parasite-rich preparations. In order to use the *in vitro* culture systems as a reliable source of parasite material, the initial work of the present study concentrated on attempts to optimise the culture system used for already-established stocks of *B.bovis* and to establish *in vitro* isolates of *B.bigemina*, some of which had not previously been established as continuously growing cultures in the past.

The attempts to increase parasitaemias of *B.bovis in vitro* were based on the incorporation of feeder cells into cultures. Unexpectedly, BAE cells did not improve parasitaemias of *B.bovis* cultures. In contrast, incorporation of MPWC into growing cultures resulted in increases of parasitaemia, although it did not improve resuscitation of cryopreserved stabilates. However, the increase achieved, although significant, was not substantial in practical terms in comparison to the results obtained with the conventional culture system used for *B.bovis* at CTVM. It appears that there is a limit for growth of *B.bovis in vitro*, beyond which it is not possible to increase the parasitaemia. Therefore the approach to increasing parasitaemias in culture by the use of feeder cells was not pursued. Thus, all material for the immunochemical characterisation of *B.bovis* stocks was obtained from iRBC cultured using minor modifications of the microaerophilous stationary phase (MASP) technique (Levy & Ristic, 1980), and further efforts were directed to achieving a method by which iRBC could be efficiently concentrated. Nevertheless, the encouraging results obtained from experiments in which MPWC were incorporated into cultures suggest they might be useful in attempts to establish new isolates from field infections.

Further achievements from the experiments on the *in vitro* cultivation of *B.bovis* described in this thesis include the re-establishment of the Mexico stock *in vitro* from peripheral blood taken from a calf when the parasitaemia was so low that no parasites were detected in Giemsa stained smears, and the replacement of NBS with immune calf sera to supplement culture media without inhibition of parasite growth. The success in re-establishing *B.bovis* in cultures

from blood with low parasitaemia can be seen as an indication that the *B.bovis* culture system used in the present study is well standardised and reliable. The lack of inhibition of *B.bovis* growth *in vitro* by sera taken from calves after infection with either *B.bovis* or *B.bigemina* (sero-positive by IFAT) shows that the stocks of *B.bovis* used in the present study are well adapted to grow *in vitro*, and also indicates that sero-positive animals in endemic areas might be suitable for provision of serum to supplement culture media. Moreover, these results indicate that the *B.bovis* culture systems might be unreliable for evaluating aspects of the bovine immune response *in vitro*.

It was more difficult to achieve a continuous supply of *B.bigemina* parasites than of *B.bovis*, since none of the culture conditions tested in the present study resulted in the establishment of the two African isolates (Zaria and Muguga) as continuous cultures. This was a severe constraint to comparisons between the antigenic profiles of different stocks of *B.bigemina*. The majority of the immunochemical studies were performed using the only available stock of *B.bigemina* (Mexico) that had previously been established *in vitro* (Vega et al, 1985a). Surprisingly, the Mexican stock of *B.bigemina* was successfully grown continuously *in vitro* for more than two months under culture conditions very similar to those which failed to even maintain parasites of the other two stocks of *B.bigemina* alive beyond 48 hours. These results, in association with many unsuccessful attempts to establish the African stocks *in vitro* prior to the present study and further recent unsuccessful attempts using different culture conditions (L.Bell-Sakyi, E.Posnett, personal communications), led to the conclusion that the two African stocks have different requirements from those of the Mexican stock of *B.bigemina,* and pointed to the need for further investigations into the *in vitro* cultivation system of *B.bigemina* parasites. Thus, the only reproducible source of *B.bigemina* parasites to which one could return regularly was the cultured Mexican stock; however, as for *B.bovis*, the *B.bigemina* culture system provided maximum parasitaemias around 5-7%, which was lower than desirable for production of parasite samples for use in the immunochemical analysis. Therefore, it was necessary to develop efficient methods for concentration of either iRBC or free parasites in order to obtain parasite-rich extracts.

The methods attempted for concentration of *Babesia* parasites in the present study showed that each species of *Babesia* should be considered separately, since techniques for concentration of *B.bovis* will not necessarily be appropriate for concentration of *B.bigemina*. For instance, the release of free merozoites into culture supernatant after CO_2 deprivation of cultures was considered to be an appropriate method for concentration of *B.bovis* but it was not found to be so efficient for *B.bigemina*. For this parasite it was found that smaller proportions of merozoites are released into the culture supernatant and, although no investigation was carried out to evaluate viability of free merozoites of *B.bigemina*, their

appearance was not as healthy as those of *B.bovis*, suggesting that they may have suffered damage during the CO_2 deprivation. The free merozoites of *B.bovis* released after CO_2 deprivation of cultures were viable, as indicated by their ability to infect new RBC in cultures. However, as differential lysis of uninfected RBC (Kahl et al, 1982a) yielded a good concentration of parasites, it was decided to use this technique to concentrate *B.bovis* for preparation of crude ELISA antigens and samples for protein electrophoresis. Differential lysis of uninfected RBC has not been successfully applied to concentration of *B.bigemina* iRBC, since they are more fragile than uninfected RBC and are lysed first (Mahoney, 1967a; Wright, 1973b). Thus, alternative techniques for concentration of iRBC using density gradient centrifugation were attempted in the present study. Amongst these the continuous density gradient generated after centrifugation at 30,000 x g for 13 minutes using a Percoll solution with density of 1.095 g/ml (68.48% of Percoll) yielded the best concentration of *B.bigemina* iRBC and therefore it was the method used for provision of parasite-rich material for the immunochemical characterisation of this parasite.

Once the techniques for concentration of each species of *Babesia* had been defined, the work was directed towards the immunochemical characterisation of both somatic components (resulting from lysis of either whole iRBC or freed parasites) and soluble exoantigens released into culture supernatants. This approach was taken hoping that specific somatic components of each parasite would be useful for the development of ELISAs for detection of antibodies produced against whole parasites. Moreover, antibodies produced against exoantigens would be useful for the development of an ELISA for detection of circulating antigens in serum of infected animals. Such a test, if highly sensitive, would be more appropriate than the detection of antibodies for identification of carrier animals with low levels of antibodies.

Prior to initiating the immunochemical analysis of *Babesia* stocks, studies were carried out with the aim of identifying the most appropriate methods for preparation of samples for electrophoresis. As far as somatic antigens were concerned, results from the present study showed that there is no need to use enzyme inhibitors for the preparation of both *B.bovis* and *B.bigemina* samples if the lysis with SDS-sample buffer is carried out immediately after harvesting iRBC or free parasites. Lysates prepared in this way remained antigenic and the antigens did not degrade when preserved at -80°C.

Regarding the immunochemical analysis of exoantigens, two steps were considered to be essential before preparation of samples for electrophoresis. The first was the removal of albumin present in the bovine serum used to supplement culture media which, due to its high concentration, disturbed the protein bands with approximately similar molecular weight when separated by SDS-PAGE. This was successfully achieved by the use of Affigel Blue, which

binds to serum albumin allowing its removal. However, results obtained from the Western immuno-blotting analysis of culture supernatants showed that perhaps more important than removal of albumin would be the removal of IgG which reacts with the anti-bovine IgG conjugate, and in the present study, appeared to be the major contaminating serum component in the culture media. The second step judged to be necessary to precede the SDS-PAGE analysis of culture supernatants, was the concentration of parasite exoantigens, enabling their detection by the usual gel staining techniques. Attempts to concentrate culture supernatant using a centrifugal concentration procedure were unsuccessful under the conditions used here. Concentration of culture supernatant was easily achieved by a simple technique based on removal of water by the use of Lyphogel.

The immunochemical analysis of both somatic components and exoantigens, which included the use of sera experimentally raised against individual stocks of *Babesia*, McAbs which had been raised against *B.bovis* and sera collected from animals in endemic areas, was based on two major techniques: Western immuno-blotting and immunoprecipitation of ^{35}S-labelled proteins. The aim of the characterisation was to identify relevant species-specific antigens present in all stocks of each *Babesia* species, which were then targeted for purification and the development of ELISAs. As a panel of McAbs produced against *B.bovis* was available, it was hoped that some of them would recognise relevant specific epitopes and therefore be useful for purification of specific antigens in an affinity chromatography procedure.

As the culture supernatants comprised complex mixtures of proteins (both parasite secreted/excreted products and serum contaminants), after albumin removal and concentration, culture supernatants were subjected to fractionation by HPLC, hoping that the proteins could be separated according to their size and iso-electric points into fractions containing less complex mixtures of proteins. This, it was hoped, would facilitate the purification of particular proteins of known molecular weight. However, results from the HPLC fractionation were disappointing and did not permit the identification of either *B.bovis* or *B.bigemina* exoantigens for use as diagnostic tools. At the end of the fractionation procedure, it was found that IgG, originating from the bovine serum component of culture media, was the major serum contaminant present in the resultant fractions, making it impossible to identify specific parasite exoantigens released into culture supernatants.

The first conclusion drawn from the immunochemical characterisation of somatic antigens of *B.bovis* stocks described in this thesis was that antigenic diversity exists amongst the stocks. Antigenic diversity of somatic antigens was observed by the two techniques used here - Western immuno-blotting and immunoprecipitation - and reinforced the need for selection of species-specific antigens which are common to all stocks of the parasite for the development of immunodiagnostic tests designed to be used in epidemiological studies world-wide.

The immunochemical analysis of *B.bovis* components included three stocks which had been originally isolated from three different continents. These were an Australian isolate (Lismore), a Mexican isolate (Mexico), and a South African isolate (Kwanyanga). Throughout the present study, the Kwanyanga and Mexico stocks showed similar antigenic profiles, whereas the Lismore stock was different from the other two with regard to some antigens. The Western immuno-blotting analysis showed that three antigens (molecular weights of 70, 39 and 37 kDa) that were present in the Kwanyanga and Mexico stocks were absent in the Lismore stock of *B.bovis*. In contrast, a 30 kDa antigen was identified as being Lismore specific, since it was not detected in the other two stocks. Thus, these antigens were not considered not to be appropriate candidates for the development of ELISAs.

It was hypothesised that antigenic differences between *B.bovis* stocks already reported by other workers (Kahl et al, 1982b; 1983; Dalrymple et al, 1992) could be due to differences in the proportions of distinct sub-populations of parasites within each stock. With this in mind, the Lismore stock was cloned *in vitro* by limiting dilution in order to compare immunochemically cloned and uncloned lines. However, no evidence of differences between the parent and cloned lines could been detected when cultured parasites were compared by *in vitro* growth rates, morphology and GPI isoenzyme patterns and, at the end of the experiment, it was assumed that the Lismore stock maintained in culture at CTVM comprises a homogeneous population of *B.bovis* parasites. This was confirmed in a recent report by Dalrymple and co-workers, who found in a molecular analysis at the DNA level that the particular Lismore isolate, from which the CTVM culture originated, comprises a homogeneous population of parasites (Dalrymple et al, 1992).

Despite the antigenic diversity observed amongst the stocks of *B.bovis* in immuno-blotting, a large number of the identified antigens fulfilled the criteria of being species-specific yet common amongst all stocks. These were identified as potential candidates for use in the development of ELISAs. These were seen at molecular weights of 185, 140, 121, 95, 76, 56, 28, 27, 18 and 14 kDa. Throughout the Western immuno-blotting analysis, the 56 kDa protein was identified as the major immunodominant antigen recognised by both experimentally raised anti-*B.bovis* sera and field sera and therefore was a potential candidate for use as an immunodiagnostic target.

The technique for immunoprecipitation of ^{35}S-methionine labelled proteins used in the present study was found to be very sensitive, and useful for identification of *Babesia* antigens both in the lysates and culture supernatants. All of the labelled material was of parasite origin, as illustrated by the lack of incorporation of ^{35}S-methionine in both lysates and supernatant of uninfected RBC cultures. However, it was found in the present study that all the steps involved in the immunoprecipitation technique needed to be carefully standardised in

order to make it a reliable tool for identification of specific *Babesia* antigens. The major constraint to this technique appeared to be related to the high non-specific background observed throughout the analysis. Thus, the inclusion of negative controls (pre-infection sera, PBS or distilled water) throughout the analysis was judged to be essential.

Four *B.bovis* species-specific somatic antigens were identified by immunoprecipitation (88, 75, 65 and 46 kDa). However they were seen at molecular weights different from those identified by Western immuno-blotting. Although some antigens had molecular weights close to some identified by Western immuno-blotting and therefore might be the same protein, results from the present study showed that antigens identified by one technique could not necessarily be correlated with those identified by the other. Thus, the four *B.bovis* species-specific somatic antigens identified by immunoprecipitation, plus many identified by Western immuno-blotting, were all candidate antigens for use in the development of *B.bovis* specific antibody detection ELISAs.

Unfortunately, none of the *B.bovis* candidate antigens were recognised by any of the McAbs when tested in either Western immuno-blotting or immunoprecipitation, despite the fact that all the McAbs reacted with *B.bovis* parasites in IFAT. Thus the approach of using one or more McAbs in an affinity column to purify relevant antigens was not pursued. The low reactivity of the McAbs in Western immuno-blotting, ELISA and immunoprecipitation, contrasting with their good reactivity in IFAT was interpreted as possibly being related to their specificity for conformational epitopes (Harlow & Lane, 1988).

Regarding the identification of *B.bigemina* antigens, the present analysis faced more limitations than for *B.bovis* stocks, particularly as a consequence of the failure to maintain continuously more than one stock of *B.bigemina in vitro*. Even that stock (Mexico) was only acquired late in the study. The only possible comparison was performed between two stocks of *B.bigemina* (Kenya and Mexico) by Western immuno-blotting. This allowed identification of two somatic antigens (80 and 65 kDa) as common to both stocks of *B.bigemina* yet absent from *B.bovis* and therefore potential candidates for use as specific immunodiagnostic targets. The analysis by immunoprecipitation of ^{35}S-labelled somatic antigens, which was performed with the only *B.bigemina* stock that could be grown *in vitro* (Mexico), allowed identification of specific antigens with a wide range of molecular weights (210 to 21 kDa) and these were also seen as potential candidates for use in the development of specific ELISAs.

In the search for exoantigens in supernatants of *B.bovis* and *B.bigemina* cultures, the immunoprecipitation of ^{35}S-labelled products proved to be an appropriate method, allowing identification of a wide range of species-specific antigens. Six *B.bovis* exoantigens (80, 72, 58, 38, 34 and 24 kDa) were shown to be common amongst the three stocks and four *B.bigemina* exoantigens (240, 112, 50 and 29 kDa) were identified as species-specific. These

were therefore potential candidates for use as immunodiagnostic targets. However, the great limitation in the use of exoantigens for the development of ELISAs, was related to the purification of relevant components from the mixture of proteins (most of which were serum contaminants) present in supernatants from cultures. The lack of success of the HPLC fractionation used in the present study in identifying *Babesia* exoantigens from culture supernatant made it difficult to purify the relevant exoantigens identified by immunoprecipitation, and therefore it was not possible to develop ELISAs with specific exoantigens.

Electroelution of proteins from acrylamide gels appeared to be an appropriate method for purification of *B.bovis* and *B.bigemina* antigens since immuno-affinity purification was not possible using the McAbs available in the present study. Some candidate somatic antigens of each *Babesia* species were therefore selected for elution and used for the development of ELISAs. Four protein bands of *B.bovis* (121, 75, 56 and 46 kDa) and four of *B.bigemina* (210, 80, 65 and 50 kDa) were successfully eluted from SDS gels. All four *B.bovis* proteins remained antigenic after elution in Western immuno-blotting; however only one (56 kDa) was found to discriminate between anti-*B.bovis* and anti-*B.bigemina* sera in ELISA and therefore it was selected for the development of a *B.bovis* specific antibody detection ELISA.

Due to the fact that none of the *B.bigemina* proteins were able to discriminate between anti-*B.bigemina* and anti-*B.bovis* sera in ELISA, although two amongst the four eluted remained antigenic in Western immuno-blotting, it was not possible to develop a *B.bigemina* specific ELISA using eluted proteins. These findings were interpreted as being possibly due to more *B.bigemina* epitopes being cross-reactive with *B.bovis* than vice-versa. Nevertheless, as results obtained from the immunochemical analysis and the ELISA checkerboard titration gave strong indications that the 56 kDa protein is a *B.bovis*-specific antigen, its use alongside a crude *B.bigemina* antigen in ELISAs should permit discrimination between the two infections in endemic areas.

The purified *B.bovis* 56 kDa protein was found to have potential in replacing crude antigenic preparations in antibody detection ELISAs, as demonstrated by the high level of agreement (at least 95%) between the two antigens in detecting *B.bovis* reactive field serum samples. Furthermore, the ability of the 56 kDa protein to detect high proportions of positive sera from cattle from three different countries (Malawi, Mozambique and Brazil) suggests that this protein is conserved amongst different isolates of *B.bovis* and therefore could be used world-wide.

The ELISA developed here using the *B.bovis* 56 kDa protein as antigen for detection of *B.bovis* specific antibodies has considerable potential for use in epidemiological studies on bovine babesiosis in Brazil, where the assay should be implemented in the near future. In

order to make it viable, the 56 kDa protein should be produced by recombinant DNA technology. The assay would then have advantages over conventional antigen preparations in several ways. In addition to eliminating cross-reactions due to anti-*B.bigemina* antibodies present in sera from cattle in endemic areas, due to concurrent infections with *B.bigemina*, the 56 kDa protein would be produced artificially, therefore eliminating batch-to-batch variations. Furthermore, there would be no need to sacrifice experimental cattle for the bulk preparation of crude antigens.

9.1 FUTURE PROSPECTS

The experiments described in this thesis report relevant achievements related to the *in vitro* cultivation, concentration and immunochemical characterisation of *B.bovis* and *B.bigemina* parasites and open new prospects for a better understanding of bovine babesiosis in tropical countries where the two diseases are endemic.

Future work should include further investigations on the 56 kDa *B.bovis* protein, used here to develop a specific antibody detection ELISA with potential for sero-epidemiological surveys. Further work should be carried out in order to produce its recombinant form, enabling its use on a large scale. A detailed investigation regarding its composition, origin and dynamics during *B.bovis* infections may reveal other features and further potential uses for this component, for instance as a protective immunogen.

Moreover, of particular relevance would be the production of antibodies against the 56 kDa protein. These antibodies could them be used to develop ELISAs for detection of either circulating antigens or immune complexes, as developed for *Trypanosoma* sp (Rae & Luckins, 1984) and *Theileria mutans* (Katende et al, 1990), which, in association with the antibody detection ELISA developed here, would probably allow more reliable detection of carrier animals than with the use of a single test. Specific antibodies produced against the 56 kDa protein would also be necessary for screening a *B.bovis* genomic library with a view to identifying the gene encoding it and subsequent production of its recombinant form.

As the major constraint of the present effort to characterise stocks of *B.bigemina* was the lack of an *in vitro* culture system which enabled continuous growth of all available *B.bigemina* isolates, future work should primarily be directed towards the improvement of the present *in vitro* culture system for *B.bigemina*. This should include investigations on the use of atmosphere with a wider range of O_2 tension and PCVs than those used in the present study to initiate cultures.

In the search for *B.bigemina* specific antigens, future work should include the use of more powerful techniques, such as two-dimensional gel electrophoresis, to resolve immune complexes which would allow more precise identification of specific antigens.

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APPENDICES

APPENDIX 1 REAGENTS AND SOLUTIONS

Giemsa Stain Solution

Glycerol (Analar)	540 ml
Methanol	840 ml
Giemsa powder (Merck)	10 g
Azur II (0.2 g/100 ml)	2.75 g

The Giemsa powder was dispensed into a large glass mortar. Some of the glycerol was added and ground throughly. The mixture was poured into a large flat-bottomed flask. Gradually the rest of the glycerol was added and the flask was heated in a 60° C water bath for 1 hour, with intermittent shaking, and then cooled to room temperature. The methanol was added and the solution stirred overnight. The Azur II was added followed by another overnight stirring. The solution was then filtered (Whatmans No 4) and stored in a dark bottle.

Giemsa Buffer (pH 7.2)

1 tablet GURR (cat No 33201) per litre of distilled water

IFAT mountant (66% glycerol)

50 mM Tris-Cl buffer (pH 9.2)	10 ml
glycerol	20 ml

McIlvane's buffer solution

$Na_2HPO_4 2H_2O$	19.66 g
citric acid	6.46 g

The solution was made up to 1 litre with distilled water and pH adjusted to 6.4 with HCl.

PIGA buffer

sodium pyruvate	50 mM
miosine	50 mM
glucose	100 mM
Na_2HPO_4	500 mM
adenosine	5 mM

The solution was made up in 0.9%(w/v) NaCl and pH adjusted to 7.2 with HCL.

SDS-PAGE solutions

SDS reducing sample buffer

solution a)	Tris	45 mg
	EDTA(disodium)	223 mg
	distilled water	10 ml
solution b)	SDS	1.0 g
	2-mercaptoethanol	0.45 ml
	glycerol	5 ml

A spatula tip of bromophenol blue was added and the solution made up to 10 ml with distilled water.

Equal volumes of a) and b) were mixed.

SDS non-reducing sample buffer

SDS	1 g
iodoactamide	95 mg
glycerol	5 ml

Ponceau-S to colour to 10 ml in distilled water

Gel Solutions

Solutions required (ml)	Concentration of gel solutions 20%	10%	7%	4.5%
Stock acrylamide (40%)	100	50	35	11.25
x 4 lower Tris	50	50	50	--
x 4 upper Tris	--	--	--	25
sucrose (g)	30	--	--	--
distilled water	20	100	115	63.75
Total volume	200*	200*	200*	100**

* frozen at -20° C as 20-ml aliquots

** frozen at -20° C as 10-ml aliquots

x 4 lower Tris

Tris	36.4 g
SDS	0.8 g
distilled water to	200 ml

pH adjusted to 8.8 with conc. HCl

x 4 upper Tris

Tris	12.12 g
SDS	0.8 g
distilled water to	200 ml

pH adjusted to 6.8 with conc. HCl

Electrophoresis and protein elution buffer

Tris	3.03 g
glycine(chr.homogeneous)	14.41 g
SDS	1.00 g

Made up to 1 litre in distilled water

PBS with protease inhibitors

PBS	199 ml
EDTA	5 mM
leucopeptin	0.2 mM
PMFS	1 mM

Western immuno-blotting solutions

Transfer buffer

electrode buffer	100 ml
methanol	100 ml
dist.water	300 ml

Blocking buffer (x 10)

Tris	50 mM
NaCl	150 mM
EDTA	1 mM
NP 40	0.05%
gelatin	0.025%
thiomersal	0.02%

made up to 1 litre and pH adjusted to 7.4 with conc. HCl. Before use, the solution was diluted 1:10 with distilled water and 5% (w/v) of dried skimmed milk (Marvel) was added.

Tris buffered saline (TBS)

Tris	20 mM
NaCl	0.5 M

made up to 2 litres and pH adjusted to 7.5 with conc. HCl.

Highly purified water

The water was first deionised in a MILI RO 60 water purification system (Millipore) and at the same time purified by reverse osmosis. The water was then given a final polishing with a MILLI Q water purification system (Millipore). The final product had a resistivity of 18 megahoms, indicating a high degree of purity.

Metabolic labelling and immunoprecipitation solutions

PBS-methionine (for washes of Sephadex column)

PBS	199 ml
PMSF	34.84 mg (dissolved in 1 ml of ethanol)
TPCK	10 mg
TLCK	5 mg
Methionine	20 mg

Lysis Buffer

NaCl	0.14 M
Tris	10 mM

the solution was made up in distilled water and pH adjusted to 7.8 with HCl. To this solution the following chemicals were added:

iodoacetamide	5 mM
PMSF	2 mM
EDTA	5 mM
sodium metabisulphite	2 mM
TLCK	0.1 mM
NP 40	1%
sodium deoxycholate	0.1%

Co-precipitation diluent

Tris	20 mM
NaCl	50 mM
NP 40	0.2%

Solution was made up in distilled water and pH was adjusted to 8.0

HPLC buffers

Elution buffer (size exclusion fractionation)

Na_2SO_4	0.1 M
KH_2PO_4	0.1 M
NaN_3	0.001 M

pH adjusted to 7.0 with HCl

Buffer A

Tris	20 mM

pH adjusted to 7.0 with HCl

Buffer B

Tris	20 mM
NaCl	150 mM

pH adjusted to 8.3 with HCl

APPENDIX 2 *IN VITRO* CULTURE RESULTS

Number of *B.bovis* infected cells in 1,000 RBC in cultures of Mexico stock with different sera (Fig 4.1).

serum	well No	days					
		1	2	3	4	5	6
calf 396 [1]	1	7	22	32	21	19	8
	2	8	16	34	24	32	10
	3	5	15	31	19	23	11
calf 397 [2]	4	6	18	13	6	9	2*
	5	11	19	17	9	6	2*
	6	5	12	22	10	5	3*
NBS	7	14	18	30	10	19	14
	8	7	15	43	20	20	19
	9	10	13	35	15	16	11

Key: * 50 fields(~250 RBC/field) counted
[1] 21 days after *B.bovis* infection
[2] 21 days after *B.bigemina* infection

Number of *B.bovis* infected cells in 1,000 RBC in cultures of Lismore stock with different calf sera (Fig 4.2).

serum	well No	days					
		1	2	3	4	5	6
NBS	1	10	30	49	27	18	37
	2	11	24	43	30	38	36
calf 396 [1]	3	9	21	41	29	32	40
	4	12	19	45	27	33	38
calf 397 [2]	5	8	29	44	30	30	41
	6	11	24	42	19	26	43
calf 399 [3]	7	9	25	46	15	28	38
	8	14	28	49	17	25	43
calf 590 [4]	9	8	30	48	16	25	32
	10	9	24	49	20	29	35

Key: [1] day 69 after *B.bovis* infection (IFAT titre 1 in 2560)
[2] day 69 after *B.bigemina* infection (IFAT titre 1 in 640)
[3] day 36 after *B.bigemina* infection (IFAT titre 1 in 2560)
[4] uninfected

Number of *B.bovis* infected cells in 1,000 RBC in cultures with MPWC or BAE as feeder cells (Fig 4.3).

condition	well n^{o}	days			
		1	2*	3	4
A	1	39	59	17	26
	2	38	54	16	30
	3	40	68	19	28
B	4	44	63	22	36
	5	41	64	19	40
	6	39	79	14	34
C	7	45	82	29	42
	8	43	72	17	27
	9	40	68	24	36
D	10	37	48	14	24
	11	29	47	11	18
	12	34	48	8	25
E	13	30	47	16	12
	14	35	58	7	18
	15	36	52	11	16

Key: A - 10^{6} MPWC B - 10^{5} MPWC
C - 10^{4} MPWC D - no feeder cells
E - BAE cells
* all wells were subcultured at 1in 5 dilution with fresh RBC suspension

Number of *B.bovis* infected cells in 1,000 RBC of established cultures of Lismore stock with MPWC as feeder cells (Fig 4.5 A).

	MPWC/ml											
	10^{4}				10^{3}				none			
days	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	5	5	5	5	5	5	5	5	5	5	5	5
2	35	29	30	26	23	28	34	26	19	18	17	15
3	64	53	48	47	60	60	58	44	37	38	34	33
3b*	4	4	4	4	5	5	5	5	5	5	5	5
4	33	31	27	26	32	37	30	31	19	25	21	26
5	52	59	64	64	60	58	57	53	48	54	49	50
6	60	85	70	79	71	78	69	89	54	60	52	42

* after subculture

Number of *B.bovis* infected cells in 1,000 RBC of established cultures of Mexico stock with MPWC as feeder cells (Fig 4.5 B).

days	MPWC/ml 10^4				10^3				none			
	1	2	3	4	1	2	3	4	1	2	3	4
1	6	6	6	6	6	6	6	6	6	6	6	6
2	34	37	30	30	31	35	38	36	19	16	21	15
3	52	71	63	49	65	52	50	47	26	48	36	33
3b*	8	8	8	8	9	9	9	9	8	8	8	8
4	45	50	49	56	55	59	45	50	30	34	43	26
5	79	101	102	109	101	85	81	93	57	79	89	50
6	80	94	81	82	81	86	101	95	80	78	82	42

* after subculture

Number of *B.bovis* infected cells in 1,000 RBC of established cultures of Kwanyanga stock with MPWC as feeder cells (Fig 4.5 C).

days	MPWC/ml 10^4				10^3				none			
	1	2	3	4	1	2	3	4	1	2	3	4
1	6	6	6	6	6	6	6	6	6	6	6	6
2	34	40	37	37	38	38	39	37	29	24	26	22
3	53	51	50	48	52	39	58	59	38	40	43	34
3b*	11	11	11	11	10	10	10	10	10	10	10	10
4	46	40	43	37	44	33	42	40	28	25	32	37
5	88	104	111	92	101	98	95	89	55	46	56	72
6	91	115	95	78	101	122	118	87	65	67	78	80

* after subculture

Proportions of different forms of *B.bovis* parasites (Lismore stock) in cultures with MPWC (Fig 4.6).

	Days														
	1			2			3			4			5		
form	10^4	10^3	Ctr	10^4	10^3	Ctr	10^4	10^3	Ctr	10^4	10^3	Ctr	10^4	10^3	Ctr
pair	78	64	63	80	91	71	73	81	64	85	73	88	22	25	20
single	17	27	30	13	6	14	26	19	27	15	27	12	57	58	51
quadr	0	0	0	1	1	0	1	0	0	0	0	0	0	0	0
picn	0	4	4	0	0	4	0	0	4	0	0	0	8	15	24
free	5	5	3	6	2	11	1	0	5	0	0	0	13	2	5

Proportions of different forms of *B.bovis* parasites (Mexico stock) in cultures with MPWC (Fig 4.7).

	Days														
	1			2			3			4			5		
form	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**
pair	62	68	62	11	13	47	68	23	20	87	28	26	24	17	13
single	36	27	34	50	50	36	29	75	77	13	72	53	54	47	46
quadr	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
picn	0	0	3	4	2	4	0	2	3	0	9	18	16	35	41
free	2	5	1	35	35	13	3	0	0	0	0	3	6	1	0

Proportions of different forms of *B.bovis* parasites (Kwanyanga stock) in cultures with MPWC (Fig 4.8).

	Days														
	1			2			3			4			5		
form	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**	10^4	10^3	**Ctr**
pair	60	56	51	3	11	6	63	39	29	12	36	28	23	17	27
single	35	36	45	69	54	33	35	51	65	73	63	59	54	65	50
quadr	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
picn	1	4	4	4	1	9	0	0	2	12	1	11	23	15	22
free	4	4	0	24	34	52	1	10	4	3	0	2	0	3	1

Number of *B.bovis* infected cells in 4,500 RBC of cultures of Lismore stock resuscitated using MPWC as feeder cells.

Days	**MPWC/ml**							
	10^4				none			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	18	11	17	17	6	12	6	8
2	35	40	54	39	37	24	34	33
3	216	133	179	174	145	139	136	156

Number of *B.bovis* infected cells in 4,500 RBC of cultures of Mexico stock resuscitated using MPWC as feeder cells.

Days	**MPWC/ml**							
	10^4				none			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	10	16	6	10	4	8	7	9
2	37	36	49	47	33	29	22	33
3	177	276	200	140	227	130	176	206

Number of *B.bovis* infected cells in 4,500 RBC in cultures of Kwanyanga stock resuscitated using MPWC as feeder cells.

	MPWC/ml							
Days	10^4				none			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
1	4	8	5	5	5	4	5	6
2	179	54	34	23	8	20	30	20
3	159	150	146	157	148	128	154	125

Number of *B.bovis* infected cells in 2,000 RBC in cultures of clone 1 (growth rate evaluation of cloned lines - Fig 4.9).

Starting parasitaemia 1%

	replicates			
days	1	2	3	4
1	45	68	35	42
2	76	66	73	86
3	98	61	41	90
4	91	74	88	60
5	63	60	52	42
6	45	48	29	36
7	31	29	34	37

Starting parasitaemia 0.1%

	replicates			
days	1	2	3	4
1*	6.2	6.6	5.9	6
2	18	34	36	14
3	54	66	41	48
4	57	61	77	60
5	57	106	102	54
6	25	71	52	62
7	85	46	21	42

Starting parasitaemia 0.01%

	replicates			
days	1	2	3	4
1*	0.6	0.6	1.1	0.6
2*	2.6	2.5	2.2	2
3	5	4	5	8
4	16	14	12	22
5	21	34	16	22
6	51	63	44	62
7	58	63	47	90

* 100 fields counted (250 cells/field) and the number of infected cells was adjusted to a total of 2,000 RBC

Number of *B.bovis* infected cells in 2,000 RBC in cultures of clone 3.

Starting	**parasitaemia 1%**			
	replicates			
days	1	2	3	4
1	54	73	82	54
2	76	70	72	80
3	84	80	78	75
4	78	72	69	87
5	33	63	53	81
6	41	58	36	39
7	70	41	71	39

Starting	**parasitaemia 0.1%**			
	replicates			
days	1	2	3	4
1*	6.3	5	5.9	7.6
2	36	26	34	30
3	59	46	37	44
4	81	46	44	73
5	75	87	96	92
6	82	101	90	74
7	78	84	29	61

Starting	**parasitaemia 0.01%**			
	replicates			
days	1	2	3	4
1*	1.3	1.1	1.5	0.7
2*	2.8	0.6	2.9	2
3	10	10	12	5
4	17	8	19	10
5	35	24	30	25
6	54	31	53	37
7	60	43	61	76

* 100 fields counted (250 cells/field) and the number of infected cells was adjusted to a total of 2,000 RBC

Number of *B.bovis* infected cells in 2,000 RBC in cultures of clone 4.

Starting	**parasitaemia 1%**			
	replicates			
days	1	2	3	4
1	30	21	42	23
2	92	78	74	69
3	116	98	108	76
4	67	56	73	63
5	67	129	114	53
6	37	73	64	59
7	32	33	42	36

Starting parasitaemia 0.1%				
		replicates		
days	1	2	3	4
1*	5.5	4.6	5.9	3.2
2	18	20	22	14
3	32	44	40	36
4	44	52	63	62
5	69	58	49	72
6	76	57	39	71
7	50	55	49	33

Starting parasitaemia 0.01%				
		replicates		
days	1	2	3	4
1*	0.9	0.4	0.6	0.6
2*	1.9	1.4	1.9	1
3	10	5	4	6
4	12	5	8	9
5	18	8	11	9
6	12	5	8	7
7	16	14	20	12

* 100 fields counted (250 cells/field) and the number of infected cells was adjusted to a total of 2,000 RBC

Number of *B.bovis* infected cells in 2,000 RBC in cultures of the Lismore parent line.

Starting parasitaemia 1%				
		replicates		
days	1	2	3	4
1	26	46	32	44
2	49	52	58	53
3	93	83	68	59
4	65	79	60	63
5	44	56	60	61
6	60	68	59	57
7	50	52	46	47

Starting parasitaemia 0.1%				
		replicates		
days	1	2	3	4
1	11	12	8	9
2	15	16	24	17
3	33	48	44	46
4	64	67	52	50
5	86	51	46	66
6	73	60	75	76
7	75	55	59	43

Starting	**parasitaemia**	**0.01%**		
		replicates		
days	1	2	3	4
1	8	5	11	9
2	14	18	17	20
3	13	9	7	10
4	16	12	13	19
5	32	20	21	31
6	53	46	56	48
7	71	76	80	88

Proportions of different forms of *B.bovis* cloned lines in cultures.

Parent line

Forms			replicates	
	1	2	3	4
pair	75	78	82	77
single	9	13	10	12
pycnotic	9	3	3	5
free	6	4	5	4

Clone 1

			replicates	
Forms	1	2	3	4
pair	73	75	65	68
single	16	13	15	20
triplet	0	2	0	1
quadrup	2	1	0	4
pycnotic	2	5	6	2
free	7	4	14	5

Clone 3

			replicates	
Forms	1	2	3	4
pair	75	70	75	72
single	19	14	11	14
triplet	2	3	1	0
quadrup	1	0	1	2
pycnotic	2	2	9	1
free	1	11	4	10

Clone 4

			replicates	
Forms	1	2	3	4
pair	72	63	62	58
single	17	17	16	21
triplet	1	0	1	1
quadrup	5	2	3	2
pycnotic	4	10	6	9
free	1	8	12	9

APPENDIX 3 PHOTOGRAPHY

Gels stained with Coomassie blue or silver stain, nitrocellulose membranes and X-ray films were photographed using a Polaroid MP4 camera (Polaroid) with type 55 POS-NEG 5 x 4 sheet film.

Coomassie blue stained gels were photographed using Wratten 11 filter, 1 sec exposure F11 and silver stained gels were photographed using Wratten 61 filter, 4 sec exposure F16, both using transmitted light. Nitrocellulose membranes were photographed using Wratten 23 filter, 0.5 sec exposure F16 using reflected light. X-ray films were photographed using Wratten 61 filter, 5 sec exposure F16, using transmitted light.

APPENDIX 4 RESULTS FROM METABOLIC LABELLING

4.1 Typical counts of radioactivity (c.p.m.) in 10 µl aliquots of fractions from ^{35}S-labelled supernatant of *B.bovis*, *B.bigemina* and uninfected RBC cultures obtained after filtration through Sephadex G25.

	Counts per minute (c.p.m.)		
Fraction number	***B.bovis* culture supernatant**	***B.bigemina* culture supernatant**	**uninfected culture supernatant**
1	77	47	23
2	90	276	32
3	101	171	21
4	928	638	51
5	13,576	1,846	439
6	45,753	7,244	1,513
7	54,954	26,602	766
8	59,974	43,251	281
9	56,609	63,762	97
10	56,932	63,888	64
11	35,522	55,934	62
12	10,238	19,705	127
13	3,521	13,722	390
14	21,972	5,239	1,460
15	55,798	33,566	8,956
16	89,544	92,697	47,127
17	122,345	476,262	102,464
18	339,562	450,394	366,903
19	470,267	236,892	459,892
20	302,129	167,233	545,273

4.2 Typical counts (c.p.m.) of samples of ^{35}S-labelled exoantigens and somatic antigens before and after TCA precipitation (pp.), and calculated percentages of incorporation.

Culture	**Type of antigen**	**Initial counts**	**After TCA pp.**	**% incorporation**
B.bovis	exoantigens	30,913	27,821	90.0
	somatic	324,561	227,193	70.0
B.bigemina	exoantigens	20,608	19,289	93.0
	somatic	335,802	239,144	71.0
uninfected RBC	exoantigens	1,499	29	2.0
	somatic	34,243	1,004	2.9

APPENDIX 5 ELISA RESULTS

5.1 Absorbance values from experimentally raised sera tested against crude *B.bovis* antigen (section 8.2.2.1)

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.= 0.310
1	198(K)	28	0.314	0.440	0.377	P
1	C11(K)	21	0.719	0.533	0.626	P
1	396(M)	28	0.541	0.507	0.524	P
1	396(M)	56	0.583	0.661	0.622	P
1	396(M)	77	0.737	0.726	0.732	P
1	396(M)	103	0.784	0.785	0.785	P
1	P49(L)	17	0.174	0.212	0.193	N
1	P78(L)	21	0.191	0.244	0.218	N
1	P78(L)	42	0.429	0.438	0.434	P
1	P78(L)	70	0.544	0.552	0.548	P
1	P78(L)	90	0.744	0.715	0.730	P
1	71X(Br)	n.k.	0.369	0.471	0.420	P
1	3334X(Br)	n.k.	0.695	0.461	0.578	P
					X= 0.522	SD= 0.190
2	399(Z)	14	0.391	0.256	0.324	P
2	399(Z)	29	0.293	0.290	0.292	N
2	399(Z)	57	0.301	0.228	0.265	N
2	399(Z)	68	0.341	0.271	0.306	N
2	399(Mug)	112	0.302	0.398	0.350	P
2	583(Z)	14	0.295	0.276	0.286	N
2	583(Z)	28	0.153	0.214	0.184	N
2	583(Z)	49	0.172	0.201	0.187	N
2	583(Mug)	104	0.284	0.250	0.267	N
2	1255(Br)	n.k.	0.226	0.269	0.248	N
2	1258(Br)	n.k.	0.230	0.242	0.236	N
2	1268(Br)	n.k.	0.286	0.278	0.282	N
2	1269(Br)	n.k.	0.310	0.274	0.292	N
2	397(Z)	28	0.259	0.165	0.212	N
2	397(Z)	49	0.238	0.201	0.220	N
2	397(Z)	83	0.172	0.184	0.178	N
2	U44 (Z)	28	0.117	0.184	0.151	N
2	7 T (Z)	34	0.088	0.082	0.085	N
2	8 T (Z)	42	0.078	0.072	0.750	N
					X= 0.233	SD= 0.080
3	396	0	0.189	0.197	0.193	N
3	399	0	0.127	0.109	0.118	N
3	583	0	0.180	0.162	0.171	N
3	397	0	0.253	0.258	0.256	N
3	P11	0	0.177	0.200	0.189	N
3	T57	0	0.255	0.256	0.256	N
3	634	0	0.255	0.175	0.215	N
3	539	0	0.178	0.165	0.172	N
3	929	0	0.123	0.130	0.127	N

Continuation

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.= 0.310
3	S126	0	0.205	0.285	0.245	N
3	37	0	0.107	0.114	0.111	N
3	V56	0	0.069	0.099	0.084	N
3	P58	0	0.167	0.264	0.215	N
3	P45	0	0.299	0.254	0.276	N
3	M13	0	0.191	0.210	0.200	N
					X= 0.188	SD= 0.061
	PBS		0.070	0.089	0.080	

Key: 1 anti- *B.bovis* serum
2 anti-*B.bigemina* serum
3 pre-infection serum

K-Kwanyanga L-Lismore M-Mexico Br-Brazil Z-Zaria Mug-Muguga
n.k. not known P.I. post-infection P- positive N- negative
X- mean SD- standard deviation
c.p.- cut-off point

5.2 Absorbance values from experimentally raised sera tested against crude *B.bigemina* antigen (section 8.2.2.2).

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.=0.689	Status c.p.= 0.574
1	198(K)	28	0.304	0.254	0.279	N	N
1	C11(K)	21	0.464	0.475	0.470	N	N
1	396(M)	28	0.410	0.438	0.424	N	N
1	396(M)	56	0.599	0.557	0.578	N	P
1	396(M)	77	0.727	0.709	0.718	P	P
1	396(M)	103	0.878	0.783	0.831	P	P
1	P49(L)	17	0.227	0.229	0.228	N	N
1	P78(L)	21	0.249	0.231	0.240	N	N
1	P78(L)	42	0.263	0.268	0.266	N	N
1	P78(L)	70	0.327	0.343	0.335	N	N
1	P78(L)	90	0.592	0.531	0.562	N	N
1	71X(Br)	n.k.	0.372	0.423	0.398	N	N
1	3334X(Br)	n.k.	0.834	0.605	0.720	P	P
					X= 0.465	SD=0.201	
2	399(Z)	14	1.169	0.992	1.081	P	P
2	399(Z)	29	1.005	0.893	0.949	P	P
2	399(Z)	57	1.19	1.024	1.107	P	P
2	399(Z)	68	1.289	1.265	1.277	P	P
2	399(Mug)	112	1.351	1.251	1.301	P	P
2	583(Z)	14	0.604	0.584	0.594	N	P
2	583(Z)	28	0.546	0.500	0.523	N	N
2	583(Z)	49	0.641	0.489	0.565	N	N
2	583(Mug)	104	1.392	1.367	1.380	P	P
2	1255(Br)	n.k.	1.052	0.880	0.966	P	P
2	1258(Br)	n.k.	1.147	1.010	1.079	P	P
2	1268(Br)	n.k.	1.157	1.065	1.111	P	P
2	1269(Br)	n.k.	0.969	0.943	0.956	P	P
2	397 (Z)	28	0.577	0.596	0.587	N	P
2	397 (Z)	49	0.885	0.913	0.899	P	P
2	397 (Z)	83	0.803	0.836	0.820	P	P
2	U44 (Z)	28	0.260	0.247	0.254	N	N
2	7 T (Z)	34	0.343	0.394	0.369	N	N
2	8 T (Z)	42	0.291	0.295	0.293	N	N
					X= 0.848	SD=0.345	
3	396	0	0.235	0.320	0.278	N	N
3	399	0	0.425	0.437	0.431	N	N
3	583	0	0.258	0.244	0.251	N	N
3	397	0	0.339	0.334	0.337	N	N
3	P11	0	0.398	0.348	0.373	N	N
3	T57	0	0.491	0.396	0.444	N	N
3	634	0	0.277	0.263	0.270	N	N
3	539	0	0.297	0.284	0.291	N	N
3	929	0	0.239	0.218	0.229	N	N
3	S126	0	0.233	0.221	0.227	N	N

Continuation

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.=0.689	Status c.p.= 0.574
3	37	0	0.293	0.226	0.260	N	N
3	V56	0	0.200	0.203	0.202	N	N
3	P58	0	0.239	0.297	0.268	N	N
3	P45	0	0.236	0.236	0.236	N	N
3	M13	0	0.279	0.236	0.258	N	N
					X= 0.290	SD=0.076	
	PBS		0.141	0.168	0.155		

Key: 1 anti- *B.bovis* serum
2 anti-*B.bigemina* serum
3 pre-infection serum

K-Kwanyanga L-Lismore M-Mexico Br-Brazil Z-Zaria Mug-Muguga
n.k. not known P.I. post-infection P- positive N- negative
X- mean SD- standard deviation
c.p.- cut-off point

5.3 Results of checkerboard titrations of anti-*B.bovis*, anti-*B.bigemina* and negative sera against eluted *B.bovis* protein bands (section 8.3.2.1).

121 kDa protein

Serum dilution	anti-*B.bovis*		anti-*B.bigemina*		pre-infection	
1:100	0.325	0.330	0.287	0.271	0.260	0.230
1:200	0.215	0.231	0.197	0.204	0.180	0.164
1:400	0.137	0.162	0.136	0.149	0.142	0.141
1:800	0.108	0.119	0.108	0.107	0.105	0.117
1:1,600	0.100	0.103	0.091	0.096	0.099	0.102

75 kDa protein

Serum dilution	anti-*B.bovis*		anti-*B.bigemina*		pre-infection	
1:100	0.283	0.296	0.234	0.242	0.210	0.187
1:200	0.182	0.189	0.173	0.174	0.142	0.160
1:400	0.146	0.144	0.140	0.127	0.128	0.132
1:800	0.125	0.115	0.106	0.116	0.129	0.122
1:1,600	0.137	0.111	0.115	0.101	0.135	0.189

56 kDa protein

Serum dilution	anti-*B.bovis*		anti-*B.bigemina*		pre-infection	
1:100	0.325	0.522	0.172	0.165	0.058	0.107
1:200	0.404	0.369	0.119	0.123	0.037	0.035
1:400	0.248	0.252	0.078	0.085	0.021	0.024
1:800	0.179	0.152	0.049	0.052	0.010	0.016
1:1,600	0.100	0.109	0.033	0.036	0.009	0.005

46 kDa protein

Serum dilution	anti-*B.bovis*		anti-*B.bigemina*		pre-infection	
1:100	0.325	0.312	0.290	0.298	0.241	0.265
1:200	0.202	0.210	0.199	0.202	0.188	0.172
1:400	0.139	0.165	0.137	0.152	0.149	0.137
1:800	0.109	0.111	0.109	0.105	0.100	0.112
1:1,600	0.100	0.102	0.100	0.095	0.101	0.100

5.4 Results of checkerboard titrations of anti-*B.bovis*, anti-*B.bigemina* and negative sera against eluted *B.bigemina* protein bands (section 8.3.2.2).

210 kDa protein

Serum dilution	anti-*B.bigemina*		anti-*B.bovis*		pre-infection	
1:100	0.148	0.167	0.168	0.149	0.044	0.050
1:200	0.099	0.106	0.086	0.082	0.020	0.026
1:400	0.062	0.066	0.056	0.057	0.009	0.013
1:800	0.040	0.033	0.039	0.031	0.004	0.008
1:1,600	0.025	0.026	0.019	0.020	0.001	0.004

80 kDa protein

Serum dilution	anti-*B.bigemina*		anti-*B.bovis*		pre-infection	
1:100	0.280	0.296	0.308	0.301	0.095	0.099
1:200	0.176	0.188	0.330	0.289	0.062	0.070
1:400	0.142	0.154	0.151	0.130	0.048	0.040
1:800	0.089	0.102	0.101	0.106	0.041	0.042
1:1,600	0.067	0.076	0.075	0.080	0.032	0.025

65 kDa protein

Serum dilution	anti-*B.bovis*		anti-*B.bigemina*		pre-infection	
1:100	0.507	0.499	0.462	0.422	0.164	0.127
1:200	0.350	0.363	0.320	0.308	0.122	0.119
1:400	0.231	0.222	0.256	0.222	0.084	0.087
1:800	0.156	0.152	0.156	0.143	0.065	0.059
1:1,600	0.109	0.105	0.115	0.111	0.056	0.051

50 kDa protein

Serum dilution	anti-*B.bigemina*		anti-*B.bovis*		pre-infection	
1:100	0.345	0.315	0.306	0.298	0.102	0.105
1:200	0.230	0.223	0.215	0.212	0.078	0.082
1:400	0.164	0.162	0.161	0.154	0.056	0.059
1:800	0.103	0.111	0.102	0.101	0.046	0.039
1:1,600	0.074	0.079	0.076	0.089	0.037	0.035

5.5 Absorbance values from experimentally raised sera tested against *B.bovis* 56 kDa protein (section 8.3.2.2).

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.= 0.352
1	198(K)	28	0.213	0.212	0.213	N
1	C11(K)	21	0.418	0.390	0.404	P
1	396(M)	28	0.328	0.326	0.327	N
1	396(M)	56	0.434	0.408	0.421	P
1	396(M)	77	0.600	0.593	0.597	P
1	396(M)	103	0.599	0.564	0.582	P
1	P49(L)	17	0.188	0.197	0.193	N
1	P78(L)	21	0.180	0.184	0.182	N
1	P78(L)	42	0.304	0.282	0.293	N
1	P78(L)	70	0.391	0.420	0.410	P
1	P78(L)	90	0.495	0.499	0.497	P
1	71X(Br)	n.k.	0.352	0.359	0.355	P
1	3334X(Br)	n.k.	0.370	0.359	0.365	P
					X= 0.372	SD=0.132
2	399(Z)	14	0.289	0.266	0.278	N
2	399(Z)	29	0.250	0.254	0.252	N
2	399(Z)	57	0.212	0.217	0.215	N
2	399(Z)	68	0.275	0.259	0.267	N
2	399(Mug)	112	0.324	0.314	0.319	N
2	583(Z)	14	0.221	0.237	0.229	N
2	583(Z)	28	0.232	0.235	0.234	N
2	583(Z)	49	0.208	0.209	0.208	N
2	583(Mug)	104	0.249	0.256	0.253	N
2	1255(Br)	n.k.	0.253	0.232	0.243	N
2	1258(Br)	n.k.	0.279	0.293	0.286	N
2	1268(Br)	n.k.	0.285	0.277	0.281	N
2	1269(Br)	n.k.	0.300	0.329	0.315	N
2	397(Z)	28	0.370	0.359	0.365	P
2	397(Z)	49	0.338	0.333	0.336	N
2	397(Z)	83	0.445	0.413	0.429	P
2	U 44 (Z)	28	0.247	0.304	0.276	N
2	7 T (Z)	34	0.237	0.209	0.223	N
2	8 T (Z)	42	0.209	0.187	0.198	N
					X= 0.275	SD=0.059
3	396	0	0.239	0.268	0.254	N
3	399	0	0.330	0.348	0.339	N
3	583	0	0.236	0.271	0.253	N
3	397	0	0.256	0.247	0.252	N
3	P11	0	0.249	0.251	0.250	N
3	T57	0	0.245	0.282	0.264	N
3	634	0	0.187	0.206	0.197	N
3	539	0	0.209	0.207	0.208	N
3	929	0	0.244	0.256	0.250	N
3	S126	0	0.271	0.257	0.264	N
3	37	0	0.226	0.223	0.225	N

Continuation

Type of serum	Calf No (stock)	Days P.I.	Well 1	Well 2	Mean	Status c.p.= 0.352
3	V56	0	0.162	0.170	0.166	N
3	P58	0	0.267	0.268	0.268	N
3	P45	0	0.390	0.345	0.368	N
3	M13	0	0.265	0.24	0.253	N
					X= 0.254	SD=0.049
	PBS		0.036	0.045	0.041	

Key: 1 anti- *B.bovis* serum
2 anti-*B.bigemina* serum
3 pre-infection serum

K-Kwanyanga L-Lismore M-Mexico Br-Brazil Z-Zaria Mug-Muguga
n.k. not known P.I. post-infection P- positive N- negative
X- mean SD- standard deviation
c.p.- cut-off point

5.6 Absorbance values from field serum samples tested against crude *B.bovis* antigen (section 8.4.2.1).

Origin	serum	well 1	well 2	mean	adj.v.	status
A	1	0.785	0.779	0.782	0.782	P
A	2	1.079	0.937	1.008	1.008	P
A	3	0.674	0.644	0.659	0.659	P
A	4	1.013	0.803	0.908	0.908	P
A	6	0.722	0.767	0.744	0.744	P
A	8	0.903	0.838	0.871	0.871	P
A	9	0.682	0.668	0.675	0.675	P
A	10	0.669	0.605	0.637	0.637	P
A	11	0.991	0.943	0.967	0.967	P
A	12	0.780	0.746	0.763	0.763	P
A	13	0.440	0.462	0.451	0.451	P
A	14	0.591	0.628	0.610	0.610	P
A	15	0.683	0.672	0.677	0.677	P
A	16	0.900	0.758	0.829	0.829	P
A	17	1.018	0.950	0.984	0.984	P
A	18	0.707	0.608	0.658	0.658	P
A	19	0.949	1.065	1.007	1.007	P
A	20	0.961	1.008	0.985	0.985	P
A	21	1.145	1.118	1.132	1.132	P
A	22	0.936	0.952	0.944	0.944	P
A	23	0.695	0.684	0.690	0.690	P
A	24	0.562	0.586	0.574	0.574	P
A	25	1.046	1.025	1.036	1.036	P
A	26	0.851	0.814	0.833	0.833	P
A	27	0.957	0.948	0.953	0.953	P
A	28	1.032	1.023	1.028	1.028	P
A	29	1.110	1.134	1.122	1.122	P
A	30	0.632	0.628	0.630	0.630	P
A	31	1.006	0.933	0.970	0.970	P
A	32	0.917	0.899	0.908	0.908	P
A	33	0.541	0.580	0.561	0.561	P
A	34	1.039	1.076	1.058	1.058	P
A	35	0.558	0.590	0.574	0.574	P
B	L210	1.460	1.455	1.458	1.069	P
B	L272	1.101	1.159	1.130	0.838	P
B	L281	0.767	0.724	0.745	0.567	P
B	L20	0.926	0.850	0.888	0.667	P
B	L244	1.032	1.003	1.018	0.759	P
B	L250	1.099	1.350	1.225	0.905	P
B	L246	1.367	1.315	1.341	0.987	P
B	L252	1.360	1.281	1.320	0.973	P
B	L270	1.098	1.108	1.103	0.819	P
B	L271	0.461	0.477	0.469	0.469	P
B	L210	1.054	1.284	1.169	1.169	P
B	L256	1.450	1.078	1.264	1.264	P
B	L190	1.366	1.507	1.437	1.437	P
B	L269	0.423	0.434	0.429	0.429	P

Continuation

Origin	serum	well 1	well 2	mean	adj.v.	status
B	L49	1.328	1.418	1.373	1.010	P
B	T200	0.518	0.513	0.516	0.404	P
B	T411	1.203	1.176	1.190	0.880	P
B	T395	1.388	1.509	1.449	1.063	P
B	T416	0.953	0.851	0.902	0.677	P
B	T387	0.998	1.004	1.001	0.747	P
B	T412	0.559	0.574	0.567	0.440	P
B	T406	1.603	1.505	1.554	1.138	P
B	T415	1.250	1.297	1.273	0.940	P
B	T413	1.094	1.071	1.083	0.805	P
B	T329	0.881	1.000	0.941	0.704	P
B	T418	0.431	0.463	0.447	0.356	N
B	L269	0.629	0.616	0.623	0.480	P
B	L45	1.412	1.251	1.331	0.980	P
B	L252	1.499	1.459	1.479	1.085	P
B	L244	0.229	0.130	0.180	0.186	N
B	L243	0.283	0.403	0.343	0.303	N
B	L278	1.133	1.202	1.168	0.889	P
B	L274	0.440	0.377	0.409	0.349	N
B	L276	1.013	1.138	1.075	0.823	P
B	T391	1.080	1.133	1.107	0.845	P
B	T409	0.532	0.428	0.480	0.400	P
B	T308	0.897	0.878	0.888	0.690	P
B	T403	0.944	0.959	0.952	0.735	P
B	T416	1.243	1.161	1.202	0.913	P
B	T414	0.292	0.253	0.273	0.253	N
B	T417	0.746	0.522	0.634	0.510	P
B	T385	1.078	1.038	1.058	0.811	P
B	T387	1.194	1.209	1.202	0.913	P
B	T389	1.663	1.568	1.616	1.207	P
B	T407	1.506	1.510	1.508	1.131	P
B	T406	1.273	1.277	1.275	0.965	P
B	T402	1.497	1.425	1.461	1.098	P
B	T405	1.313	1.423	1.368	1.031	P
B	T408	1.432	1.535	1.484	1.114	P
C	8655	0.665	0.675	0.670	0.963	P
C	4260	0.962	0.751	0.857	1.248	P
C	4320	0.522	0.616	0.569	0.809	P
C	3406	0.727	0.511	0.619	0.885	P
C	2980	0.879	1.048	0.964	0.964	P
C	7294	1.205	1.278	1.242	1.242	P
C	3334	0.695	0.755	0.725	0.725	P

Key: A - serum sample from Mozambique
B - serum sample from Malawi
C - serum sample from Brazil
adj.v. - adjusted value
P - positive
N - negative

5.7 Absorbance values from field serum samples tested against *B.bigemina* crude antigen (section 8.4.2.2).

Origin	serum	well 1	well 2	mean	adj.v.	status
A	1	0.726	0.539	0.633	0.539	N
A	2	0.729	0.720	0.725	0.616	P
A	3	0.404	0.458	0.431	0.372	N
A	4	0.683	0.740	0.712	0.605	P
A	6	0.778	0.855	0.817	0.692	P
A	8	0.887	0.917	0.902	0.763	P
A	9	0.709	0.727	0.718	0.610	P
A	10	0.681	0.728	0.705	0.599	P
A	11	0.858	0.810	0.834	0.706	P
A	12	0.809	0.869	0.839	0.711	P
A	13	0.485	0.480	0.483	0.415	N
A	14	0.613	0.555	0.584	0.499	N
A	15	0.519	0.501	0.510	0.438	N
A	16	0.854	0.835	0.845	0.715	P
A	17	0.866	0.821	0.844	0.714	P
A	18	0.519	0.715	0.617	0.526	N
A	19	0.870	0.734	0.802	0.690	P
A	20	0.838	0.774	0.806	0.683	P
A	21	1.106	1.004	1.055	0.890	P
A	22	0.918	0.911	0.915	0.773	P
A	23	0.522	0.553	0.538	0.460	N
A	24	0.565	0.627	0.596	0.509	N
A	25	0.931	0.832	0.882	0.746	P
A	26	0.847	0.934	0.891	0.753	P
A	27	0.861	0.853	0.857	0.726	P
A	28	0.873	0.927	0.900	0.761	P
A	29	1.028	1.091	1.060	0.894	P
A	30	0.782	0.780	0.781	0.662	P
A	31	0.814	0.834	0.824	0.698	P
A	32	0.857	0.978	0.918	0.776	P
A	33	0.806	0.791	0.799	0.677	P
A	34	1.087	1.100	1.094	0.922	P
A	35	0.869	0.884	0.877	0.742	P
B	L210	0.994	0.980	0.987	0.833	P
B	L272	1.080	1.096	1.088	0.917	P
B	L281	0.849	0.848	0.849	0.718	P
B	L20	1.192	1.172	1.182	0.995	P
B	L244	1.920	1.083	1.502	1.260	P
B	L250	0.928	0.945	0.937	0.791	P
B	L246	1.127	1.137	1.132	0.954	P
B	L252	1.254	1.268	1.261	1.061	P
B	L270	1.111	1.078	1.095	0.923	P
B	L271	0.783	0.782	0.783	0.664	P
B	L239	0.865	0.903	0.884	0.748	P
B	L256	1.061	1.058	1.060	0.894	P
B	L190	1.025	0.979	1.002	0.846	P
B	L269	1.046	1.027	1.037	0.874	P
B	L49	1.208	1.153	1.181	0.994	P

Continuation

Origin	serum	well 1	well 2	mean	adj.v.	status
B	T200	1.281	1.245	1.263	1.062	P
B	T411	0.984	1.008	0.996	0.841	P
B	T395	0.916	0.960	0.938	0.793	P
B	T416	0.965	0.932	0.949	0.801	P
B	T387	1.076	1.108	1.092	0.921	P
B	T412	0.853	0.872	0.863	0.730	P
B	T406	1.264	1.288	1.276	1.073	P
B	T415	1.157	1.105	1.131	0.953	P
B	T413	0.507	0.529	0.518	0.444	N
B	T329	0.752	0.758	0.755	0.641	P
B	T418	0.924	0.908	0.916	0.774	P
B	L269	0.616	0.580	0.598	0.511	N
B	L45	1.072	1.085	1.079	0.909	P
B	L252	1.040	1.053	1.047	0.883	P
B	L244	0.610	0.626	0.618	0.618	P
B	L243	0.324	0.322	0.323	0.323	N
B	L278	1.062	1.067	1.065	1.065	P
B	L274	1.109	1.092	1.101	1.101	P
B	L276	0.897	0.865	0.881	0.881	P
B	T391	1.060	1.079	1.070	1.070	P
B	T409	0.607	0.672	0.640	0.640	P
B	T308	1.173	1.262	1.218	1.218	P
B	T403	0.784	0.799	0.792	0.792	P
B	T416	0.891	1.029	0.960	0.960	P
B	T414	0.901	0.922	0.912	0.912	P
B	T417	1.093	1.135	1.114	1.114	P
B	T385	0.917	0.925	0.921	0.921	P
B	T387	1.315	1.340	1.328	1.328	P
B	T389	1.461	1.516	1.489	1.489	P
B	T407	1.454	1.460	1.457	1.457	P
B	T406	1.401	1.337	1.369	1.369	P
B	T402	1.364	1.362	1.363	1.363	P
B	T405	0.929	0.896	0.913	0.913	P
B	T408	1.429	1.546	1.488	1.488	P
C	8655	1.706	1.710	1.708	1.708	P
C	4260	1.577	1.536	1.557	1.557	P
C	4320	1.351	1.302	1.327	1.327	P
C	3406	1.257	1.132	1.195	1.195	P
C	2980	1.062	1.083	1.073	1.073	P
C	7294	0.576	0.549	0.563	0.563	P
C	3334	0.333	0.554	0.444	0.444	N

Key: A - serum sample from Mozambique
B - serum sample from Malawi
C - serum sample from Brazil
adj.v. - adjusted value
P - positive
N - negative

5.8 Absorbance values from field serum samples tested against *B.bovis* 56 kDa protein (section 8.4.2.1).

Origin	serum	well 1	well 2	mean	adj.v.	status
A	1	0.533	0.556	0.545	0.545	P
A	2	0.882	0.806	0.844	0.844	P
A	3	0.435	0.428	0.432	0.432	P
A	4	0.650	0.647	0.649	0.649	P
A	6	1.163	1.630	1.397	1.397	P
A	8	0.589	0.602	0.596	0.596	P
A	9	0.533	0.590	0.562	0.562	P
A	10	0.735	0.817	0.776	0.776	P
A	11	0.883	0.825	0.854	0.854	P
A	12	1.130	1.096	1.113	1.113	P
A	13	0.607	0.622	0.615	0.615	P
A	14	0.595	0.569	0.582	0.582	P
A	15	0.470	0.454	0.462	0.462	P
A	16	0.573	0.582	0.578	0.578	P
A	17	0.916	0.979	0.948	0.948	P
A	18	0.745	0.759	0.752	0.752	P
A	19	0.762	0.791	0.777	0.777	P
A	20	0.585	0.553	0.569	0.569	P
A	21	0.772	0.805	0.789	0.789	P
A	22	0.984	0.956	0.970	0.970	P
A	23	0.514	0.491	0.503	0.503	P
A	24	0.550	0.615	0.583	0.583	P
A	25	1.148	0.584	0.866	0.866	P
A	26	0.773	0.75	0.762	0.762	P
A	27	0.965	0.924	0.945	0.945	P
A	28	0.616	0.615	0.616	0.616	P
A	29	1.031	1.017	1.024	1.024	P
A	30	0.781	0.767	0.774	0.774	P
A	31	1.187	1.223	1.205	1.205	P
A	32	1.022	1.007	1.015	1.015	P
A	33	0.624	0.623	0.624	0.624	P
A	34	0.841	0.845	0.843	0.843	P
A	35	0.649	0.665	0.657	0.657	P
B	L210	1.346	1.303	1.325	1.132	P
B	L272	0.779	0.776	0.778	0.692	P
B	L281	0.401	0.402	0.402	0.389	P
B	L20	0.769	0.781	0.775	0.690	P
B	L244	0.87	0.852	0.861	0.759	P
B	L250	0.707	0.683	0.695	0.625	P
B	L246	1.007	0.995	1.001	0.872	P
B	L252	0.741	0.787	0.764	0.681	P
B	L270	0.696	0.699	0.698	0.627	P
B	L271	0.799	0.877	0.838	0.74	P
B	L239	0.579	0.576	0.578	0.531	P
B	L256	0.849	0.903	0.876	0.771	P
B	L190	0.901	0.943	0.922	0.808	P
B	L269	0.574	0.546	0.560	0.517	P
B	L49	0.933	0.88	0.907	0.796	P

Continuation

Origin	serum	well 1	well 2	mean	adj.v.	status
B	T200	0.710	0.709	0.710	0.637	P
B	T411	0.950	0.926	0.938	0.821	P
B	T395	0.986	0.799	0.893	0.784	P
B	T416	0.550	0.615	0.583	0.535	P
B	T387	0.515	0.545	0.530	0.492	P
B	T412	0.592	0.622	0.607	0.554	P
B	T406	0.904	0.941	0.923	0.808	P
B	T415	0.726	0.753	0.740	0.661	P
B	T413	0.719	0.709	0.714	0.641	P
B	T329	1.145	1.088	1.117	0.965	P
B	T418	0.833	0.872	0.853	0.752	P
B	L269	0.392	0.358	0.375	0.368	P
B	L45	0.537	0.572	0.555	0.512	P
B	L252	0.587	0.575	0.581	0.534	P
B	L244	0.224	0.231	0.228	0.253	N
B	L243	0.317	0.271	0.294	0.322	N
B	L278	0.893	0.726	0.810	0.869	P
B	L274	0.684	0.686	0.685	0.739	P
B	L276	0.503	0.785	0.644	0.687	P
B	T391	0.748	0.638	0.693	0.738	P
B	T409	0.592	0.333	0.463	0.498	P
B	T308	1.049	0.933	0.991	1.049	P
B	T403	0.558	0.779	0.669	0.713	P
B	T416	1.258	1.403	1.331	1.403	P
B	T414	0.285	0.298	0.292	0.320	N
B	T417	0.748	0.749	0.749	0.796	P
B	T385	0.582	0.443	0.513	0.550	P
B	T387	0.805	0.628	0.717	0.763	P
B	T389	1.311	1.182	1.247	1.316	P
B	T407	0.904	0.841	0.873	0.926	P
B	T406	1.067	1.016	1.042	1.102	P
B	T402	0.780	0.769	0.775	0.823	P
B	T405	0.559	0.605	0.582	0.623	P
B	T408	0.577	0.565	0.571	0.611	P
C	8655	0.478	0.464	0.471	0.774	P
C	4260	0.430	0.541	0.486	0.803	P
C	4320	0.337	0.374	0.356	0.540	P
C	3406	0.390	0.432	0.411	0.652	P
C	2980	0.802	0.843	0.823	0.823	P
C	7294	0.652	0.632	0.642	0.642	P
C	3334	0.578	0.603	0.591	0.591	P

Key: A - serum sample from Mozambique
B - serum sample from Malawi
C - serum sample from Brazil
adj.v. - adjusted value
P - positive
N - negative

APPENDIX 6 PUBLICATIONS

6.1 Passos, L.M.F. & Melrose, T.R. (1991) Immunochemical and biochemical diversity in stocks of *Babesia bovis*. *Proceedings of the IV International Congress on Malaria and Babesiosis*, Rio de Janeiro, Brazil.

IV INTERNATIONAL CONGRESS ON MALARIA AND BABESIOSIS
13 TO 17th AUGUST, 1991; RIO DE JANEIRO - BRAZIL

IMMUNOCHEMICAL AND BIOCHEMICAL DIVERSITY IN STOCKS OF BABESIA BOVIS **1.20**

L.M.F. PASSOS & T.R. MELROSE

University of Edinburgh, Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, EH25 9RG Scotland

With the objective of identifying immunodominant proteins to be used for diagnostic purposes, three stocks of B. bovis (from Australia, Mexico and South Africa) were cultured in vitro and analysed by SDS-PAGE, Western Blotting and Glucose Phosphate Isomerase(GPI) isoenzyme patterns. Several common immunodominant proteins were identified(relative molecular weights of 191, 159, 128, 56, 25 and 20 kDa). Sera raised against the South African and Mexican stocks did not recognize a 30 kDa protein present in the Australian stock. A 37 kDa protein present in the South African and Mexican stocks was absent in the Australian stock. The presence of GPI was confirmed in all three stocks by starch gel electrophoresis. However the Australian lysate showed a banding pattern different from the Mexican and South African stocks. The protein polymorphism and the GPI profiles will be useful in discrimination between strains of B.bovis and point to the need for careful selection of antigens for use in detection of bovine Babesia spp.

6.2 Passos, L.M.F. (1993) Identification of immunodominant *Babesia bovis* exoantigens. *Trans R Soc Trop Med Hyg* 87, 122.

Royal Society of Tropical Medicine and Hygiene
Scottish Branch
Laboratory Meeting, Edinburgh, 13 May 1992

Identification of immunodominant *Babesia bovis* exoantigens

L. Passos *Centre for Tropical Veterinary Medicine, University of Edinburgh, Easter Bush, Roslin, Midlothian, EH25 9RG, UK*

Bovine babesiosis caused by *Babesia bovis* and *B. bigemina* is one of the most common cattle tick-borne diseases in tropical and sub-tropical regions of the world and causes serious production losses.

With the objective of identifying species-specific immunodominant proteins for diagnostic purposes, 3 stocks of *B. bovis* from Australia, Mexico and South Africa were cultured *in vitro* and analysed by immunoprecipitation of ^{35}S-radiolabelled proteins present in supernatants. Using bovine immune sera experimentally raised against *B. bovis* and *B. bigemina* stocks and field sera from Brazil, where both infections are endemic, 4 specific and immunodominant proteins were identified (molecular masses of 72, 58, 38 and 24 kDa). The supernatants are also being analysed by Western blotting and the proteins will be purified and used to develop a *B. bovis* specific enzyme-linked immunosorbent assay.

6.3 Passos, L.M.F.; Brown, C.G.D.; Jones,T.W.; Bell-Sakyi, L.; Boid, R. (1993) The development of specific ELISAs for use in epidemiological studies of bovine babesiosis in Brazil. *Proceedings of the 14th International Conference of the World Association for the Advancement of Veterinary Parasitology*, Cambridge.

THE DEVELOPMENT OF SPECIFIC ELISAS FOR USE IN EPIDEMIOLOGICAL STUDIES OF BOVINE BABESIOSIS IN BRAZIL

L. M. F. PASSOS, C. G. D. BROWN, T. W. JONES, L. BELL-SAKYI AND R. BOID

Centre for Tropical Veterinary Medicine, Easter Bush, Roslin, Midlothian, Scotland

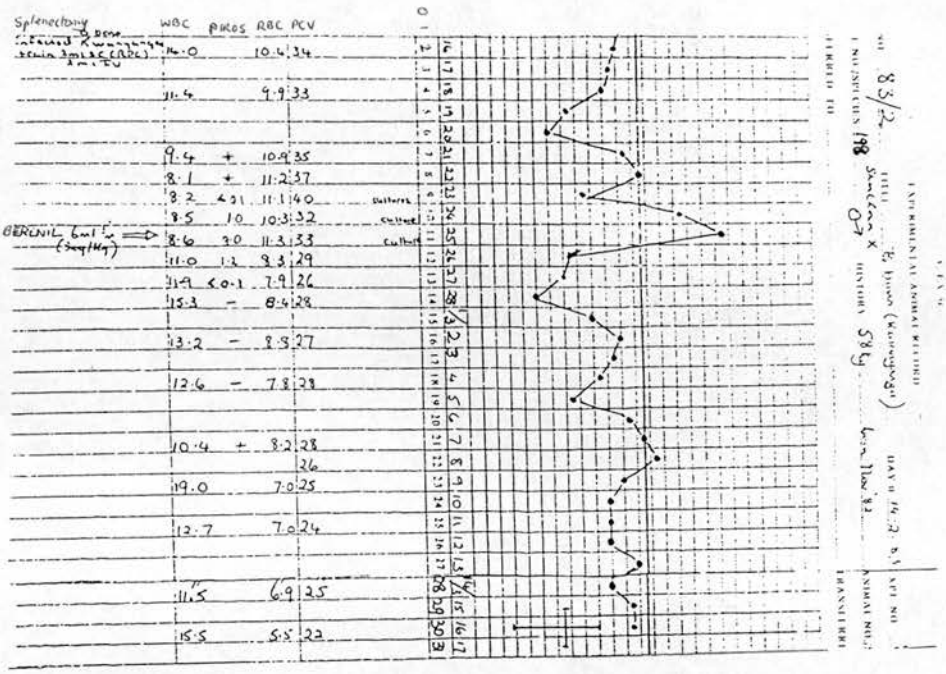
Babesia bovis and *B. bigemina*, the causative agents of bovine babesiosis in tropical areas where the tick vector (*Boophilus microplus*) occurs, are responsible for important economic losses in both milk and beef production in Brazil.

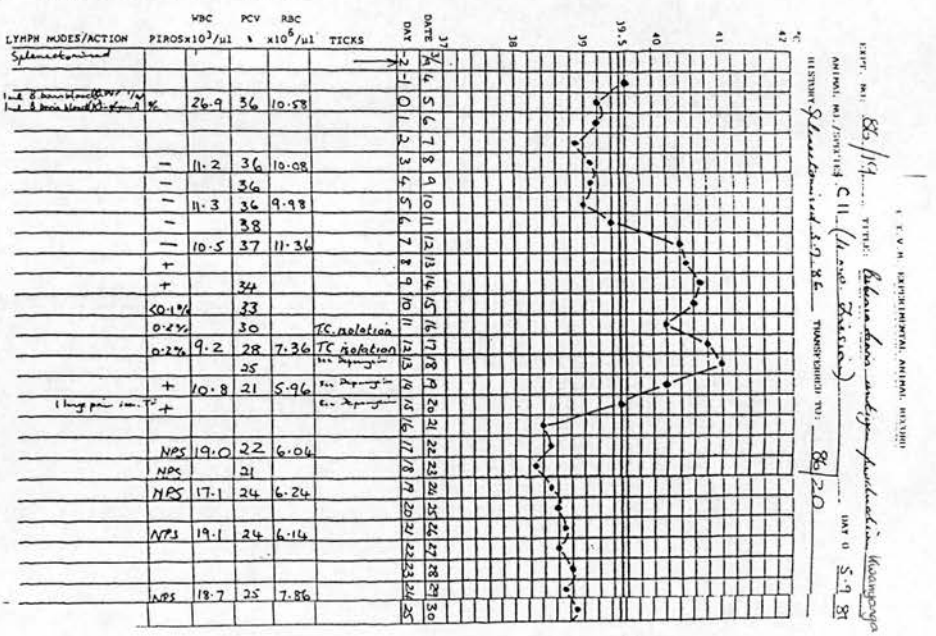
Following previous work on the identification of *B. bovis* and *B. bigemina* specific antigens using immunoblotting and immunoprecipitation of biosynthetically labelled parasite components (Passos, L.M.F. 1992 *Trans. R. Soc. Trop. Med. Hyg.* 87, 122), the present study reports the purification of selected parasite proteins from acrylamide gels and their use in the development of antibody-detection ELISAs for these two protozoans to use in epidemiological studies in Brazil. Crude antigen extracts of each parasite were compared in ELISAs with one candidate purified protein from each parasite to test against a panel of calf sera experimentally raised to three geographically different isolates of *B. bovis* and three of *B. bigemina*. In addition, these ELISA antigens were tested against a panel of sera from cattle in Brazil, Malawi and Mozambique which had been infected naturally by ticks in the field.

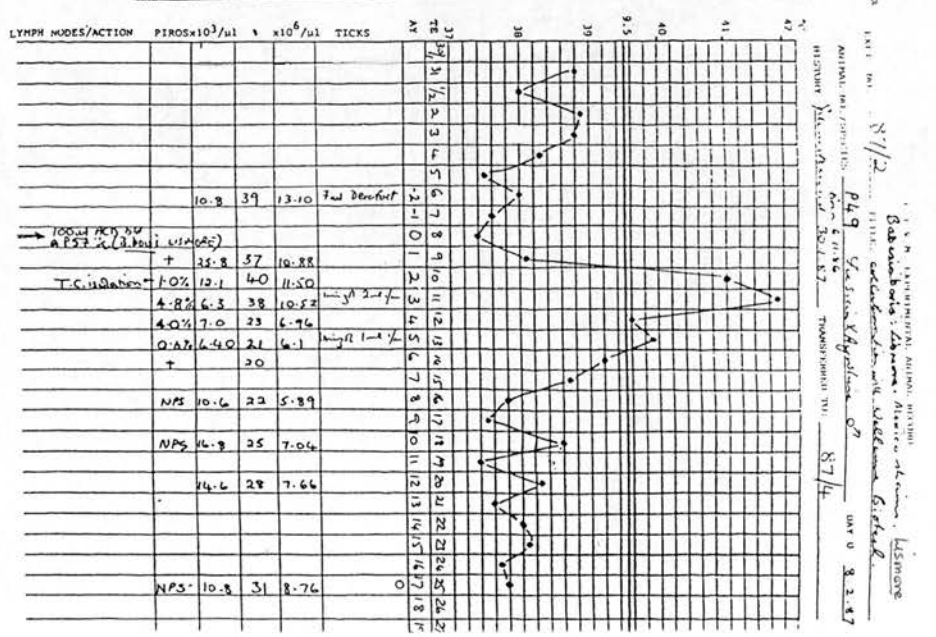
The purified *B. bovis* protein detected specific antibodies and is a potential candidate to replace the crude parasite extract in immunodiagnostic assays. However the purified *B. bigemina* protein cross-reacted with *B. bovis* antisera and other candidate proteins will need to be assessed.

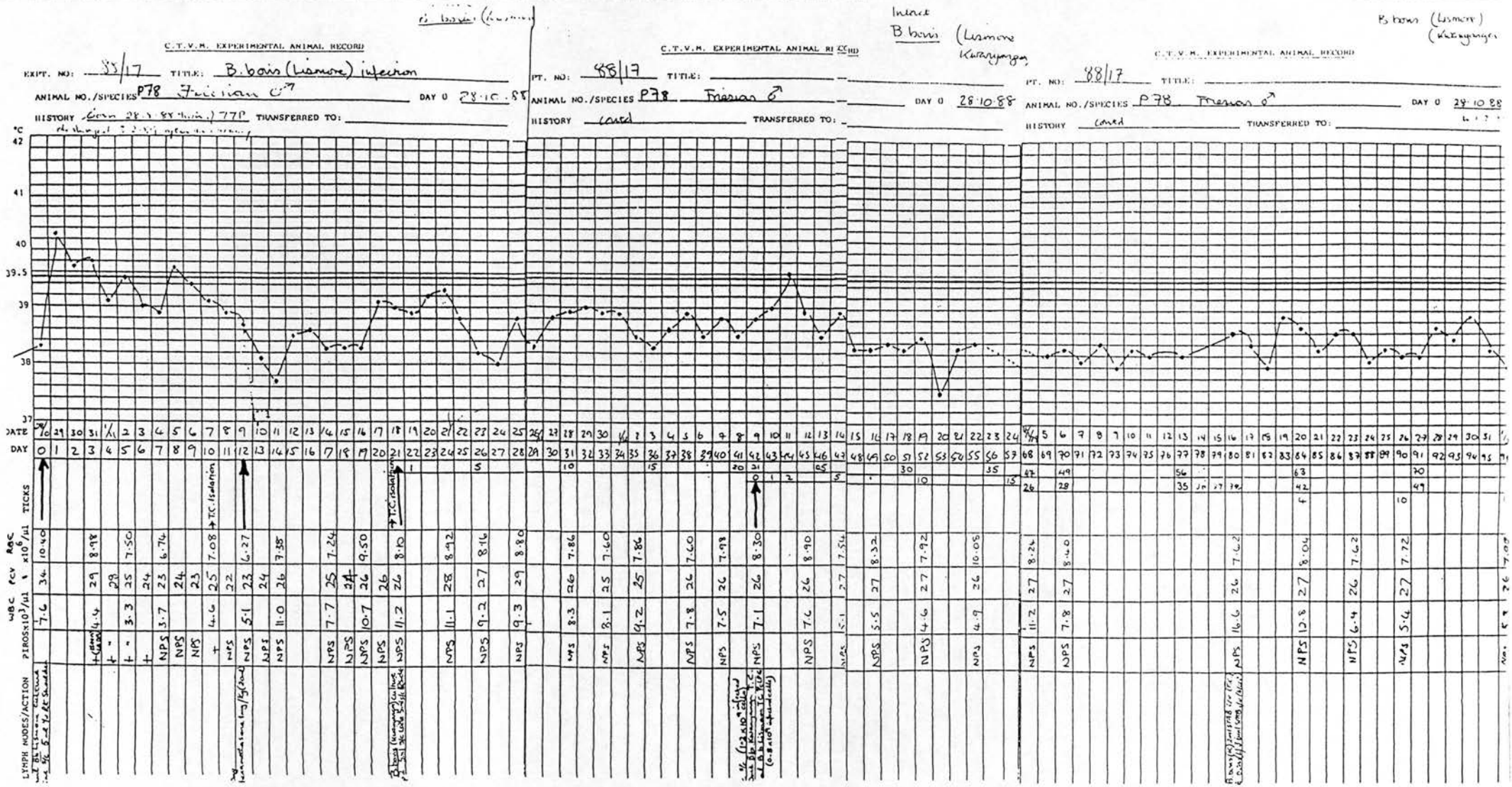
APPENDIX 7

CLINICAL AND PARASITOLOGICAL RECORDS OF CALF INFECTIONS


Splenectomy
WBC PIROS RBC PCV
14·0 10·4 34
11·4 9·9 33
9·4 + 10·9 35
8·1 + 11·2 37
8·2 4 31 11·1 40
8·5 10 10·3 32
8·6 20 11·3 33
11·0 13 9·3 29
11·9 <0·1 7·9 26
15·3 - 8·4 28
13·2 - 8·5 27
12·6 - 7·8 28
10·4 + 8·2 28
26
19·0 7·0 25
12·7 7·0 24
11·5 6·9 25
15·5 5·5 22
BERENIL 6ml I.v. (3mg/kg)
Buttocks cultured
Buttocks cultured
DAY 0 14.2.84
DATE 1 Nov 82
58kg
8.3.84
198
Sanborn x
Brahman (Washington)
EXPERIMENTAL ANIMAL UNIT
TRANSFERRED TO
ANIMAL NOS.
EXPT. NO.


LYMPH NODES/ACTION
WBC PIROS×10³/µl
PCV %
RBC ×10⁶/µl
TICKS
Splenectomised
26·9 36 10·58
11·2 36 10·08
36
11·3 36 9·98
38
10·5 37 11·36
34
33
30
TC. isolation
9·2 28 7·36
25
10·8 21 5·96
NPS 19·0 22 6·04
NPS 21
NPS 17·1 24 6·24
NPS 19·1 24 6·14
NPS 18·7 25 7·86
DAY
DATE
TEMP °C
DAY 0 5.9.84
TRANSFERRED TO 86/20
Splenectomised 5.9.84
EXPT. NO. 83/19
ANIMAL NO./IDENTITY C 11 (Nanno Tsetse)
HISTORY


LYMPH NODES/ACTION
PIROS×10³/µl
PCV %
RBC ×10⁶/µl
TICKS
10·8 39 13·10 7ml Desorbit
100ml ACD 4d
APS2 2% (3 bull (1:4 ACD)
35·8 37 10·88
T.C. isolation 1·0% 12·1 40 11·50
4·8% 6·3 38 10·52
4·0% 7·0 23 6·96
0·4% 6·40 21 6·1
20
NPS 10·6 22 5·89
NPS 16·8 25 7·04
14·6 28 7·66
NPS 10·8 31 8·76
DAY
DATE
TEMP °C
DAY 0 8.2.84
TRANSFERRED TO 87/4
Babesiosis: Lapenn, Alsace strain, Lusinge
P49 Splenectomised 30.1.84
EXPT. NO. 87/2
ANIMAL NO./IDENTITY
HISTORY


C.T.V.M. EXPERIMENTAL ANIMAL RECORD
EXPT. NO: 88/17 TITLE: B.bovis (Lismore) infection
ANIMAL NO./SPECIES P78 Friesian ♂ DAY 0 28.10.88
HISTORY from 28.3.88 (Hong) 77P TRANSFERRED TO:
C.T.V.M. EXPERIMENTAL ANIMAL RECORD
EXPT. NO: 88/17 TITLE: Infect B bovis (Lismore Kirrawee)
ANIMAL NO./SPECIES P78 Friesian ♂ DAY 0 28.10.88
HISTORY cured TRANSFERRED TO:
C.T.V.M. EXPERIMENTAL ANIMAL RECORD
EXPT. NO: 88/17 TITLE: B bovis (Lismore) (Kirrawee)
ANIMAL NO./SPECIES P78 Friesian ♂ DAY 0 28.10.88
HISTORY cured TRANSFERRED TO:
°C
42
41
40
39.5
39
38
37
DATE
DAY
TICKS
WBC PCV AGC
PIROS x10³/µl
x10⁶/µl
LYMPH NODES/ACTION
TC isolation
TC isolation

[redacted]

B bovis (Mamco)!

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: ~~90/9~~ TITLE: Production of immune serum against B.bovis and B.bigemina

ANIMAL NO./SPECIES: 396 ♂ Ayrshire DAY 0: 20.4.90

HISTORY: born 16.11.89 Rhydlewis TRANSFERRED TO: 90/9A

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

PT. NO: 90/9A TITLE: Homologous challenge of B bovis immune calf 396

ANIMAL NO./SPECIES: 396 ♂ Ayrshire DAY 0: 18.5.90

HISTORY: ex 90/9 TRANSFERRED TO: 90/9A1

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

PT. NO: 90/9A1 TITLE: Second homologous challenge with B.bovis

NO./SPECIES: 396 DAY 0: 15.6.90

HISTORY: 90/9, 90/9A. TRANSFERRED TO:

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

PT. NO: 90/9A1 (cont'd) TITLE: Third homologous challe... Second homologous challe...

ANIMAL NO./SPECIES: 396

HISTORY: 90/9, 90/9A TRANSFERRED TO: dest...

°C

42
41
40
39.5
39
38
37

DATE / DAY

DATE	29/4	30	1/5	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18

DATE	19/5	20	21	22	23	24	25	26	27	28	29	30	31	1/6	2	3	4	5	6	7
DAY	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19

DATE	8	9	10	11	12	13	14	15/6	16	17	18	19	20	21	22	23	24	25	26	27
DAY	20	21	22	23	24	25	26	27	0	1	2	3	4	5	6	7	8	9	10	11

DATE	28	29	30	1/7	2	3	4	5	6	7	8	9	10	11	12	13	14/7	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	1/8	2
DAY	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48

	PCV	RBC	TICKS
		x10⁶/µl	
29/4	35	10.64	
30	40	11.64	
1/5	38	10.48	
2	36	10.48	
3	34	9.22	
4	32	9.16	
5	31		
6	31	9.00	
7			
8			
9	28	7.80	TC/ovinum Romanowski
10	28		
11	29	9.16	
12			
13	28	8.30	
14	28	8.52	
15	29	8.68	
16			
17	28	8.50	
18	31	8.92	
19/5	31	9.02	
20			
21			
22			
23	29	8.20	
24	30	8.42	
25			
26			
27	32	9.12	
28			
29	30	8.56	
30			
31	31	8.82	
1/6			
2			
3	33	8.78	
4			
5	31	8.46	
6			
7			
8	31	8.80	
9			
10	29	8.38	
11			
12	28	7.78	
13			
14			
15/6	29	7.90	
16			
17	30	8.56	
18			
19	29	8.52	
20			
21	30	8.66	
22			
23			
24			
25	28	8.28	
26			
27	25	6.72	
28			
29	27	7.24	
30			
1/7			
2			
3	27	7.38	
4			
5	30	7.72	
6			
7	27	7.80	
8			
9	29	8.54	
10			
11	29	8.24	
12			
13	29	8.52	
14/7			
15			
16	30	8.70	
17			
18	29	8.00	
19			
20	30	8.52	
21			
22			
23	27	8.24	
24			
25	29	9.18	
26			
27	27	8.80	
28			
29			
30	27	8.20	
31			
1/8	25	7.40	
2			

	RBC	PIROS×10³/µl	LYMPH NODES/ACTION
29/4	15.8	—	
30		—	
1/5		—	
2		—	
3		—	
4		—	
5		—	
6		—	
7		—	
8		—	
9		—	
10		—	
11		—	
12		—	
13		—	
14		—	
15/5	11.4	—	
16		—	
17		—	
18		—	
19		—	
20		—	
21		—	
22		—	
23		—	
24		—	
25		—	
26		—	
27		—	
28		—	
29		—	
30		—	
31		—	
1/6		—	
2		—	
3		—	
4		—	
5		—	
6		—	
7		—	
8		—	
9		—	
10		—	
11		—	
12		—	
13		—	
14		—	
15/6	12.9	—	
16		—	
17	14.9	—	
18		—	
19	13.4	—	
20		—	
21	13.3	—	
22		—	
23		—	
24		—	
25	10.6	—	
26	10.8	—	
27	12.6	—	
28		—	
29		—	
30		—	
1/7		—	
2		—	
3	13.2	—	
4		—	
5	15.6	—	
6		—	
7	12.5	—	
8		—	
9	12.7	—	
10		—	
11	13.7	—	
12		—	
13	13.33	—	
14/7		—	
15		—	
16	11.5	—	
17		—	
18	8.0	—	
19		—	
20	13.3	—	
21		—	
22		—	
23	11.6	—	
24		—	
25	10.4	—	
26		—	
27	11.2	—	
28		—	
29		—	
30	11.9	—	
31		—	
1/8	15.2	—	
2		—	

LYMPH NODES/ACTION: B.bovis (Mamco) Scattered Sm. Red Blood cells, etc.

KILLED

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

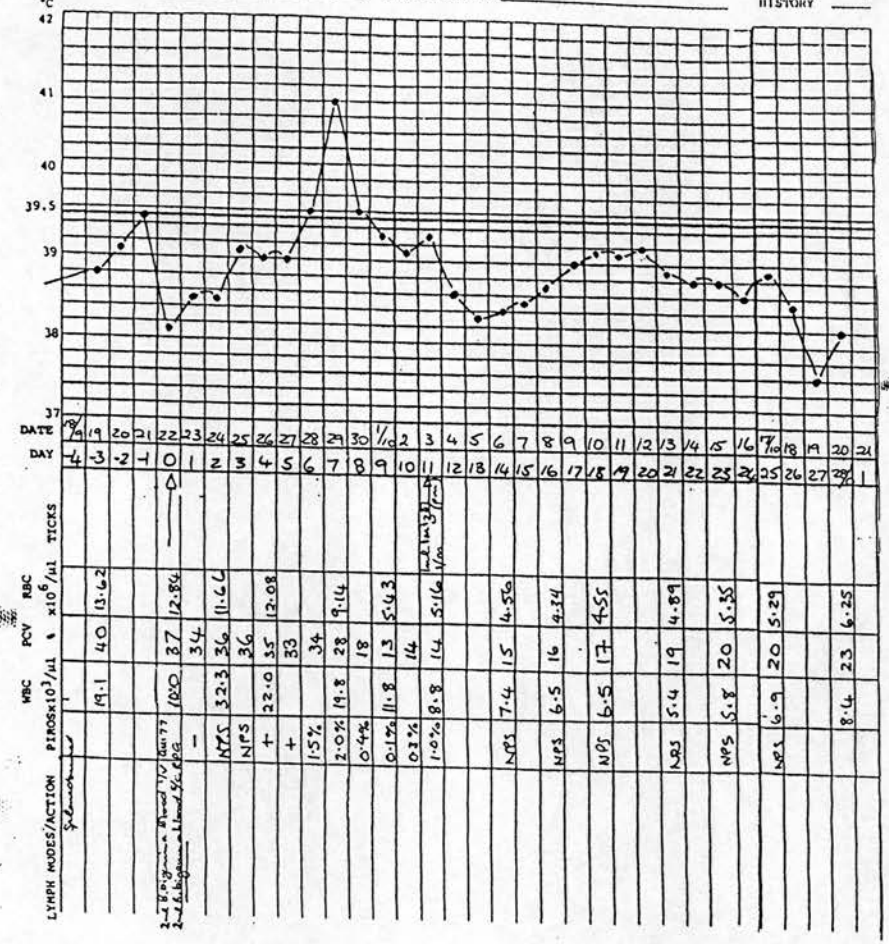
Zaria

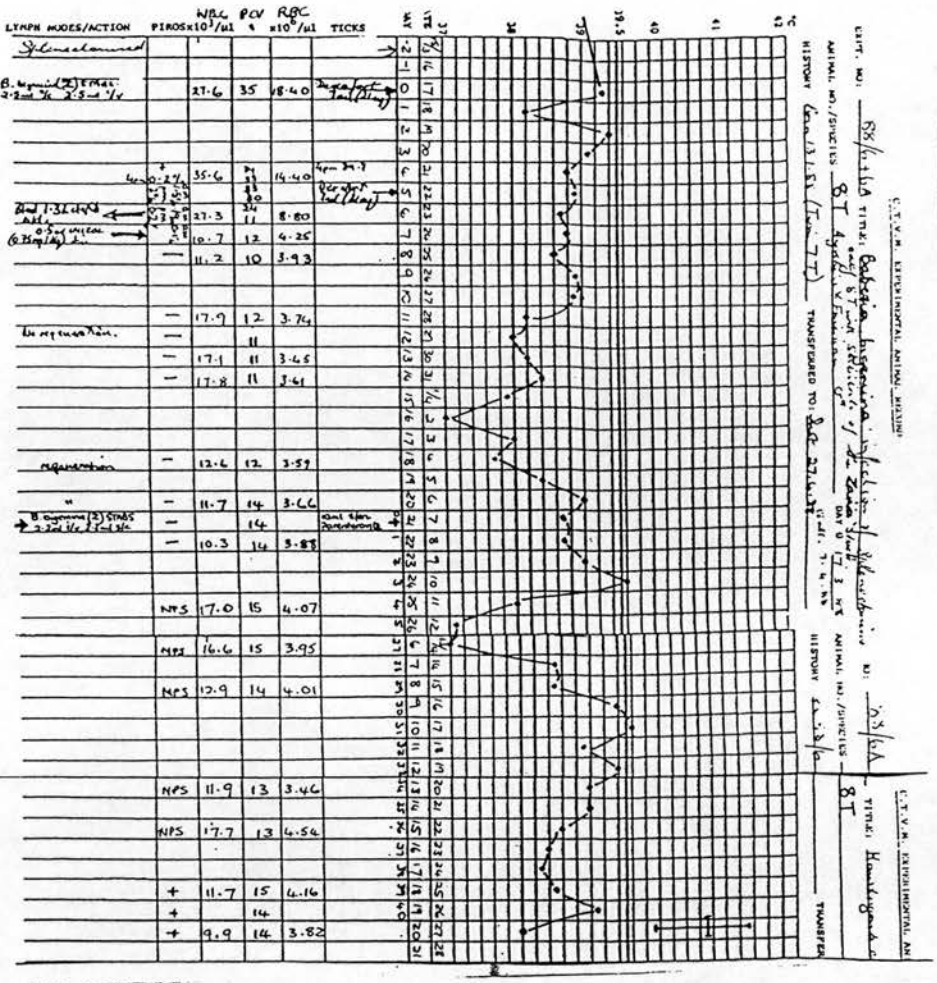
EXPT. NO: 86/198 TITLE: Babesia bigemina antigen production

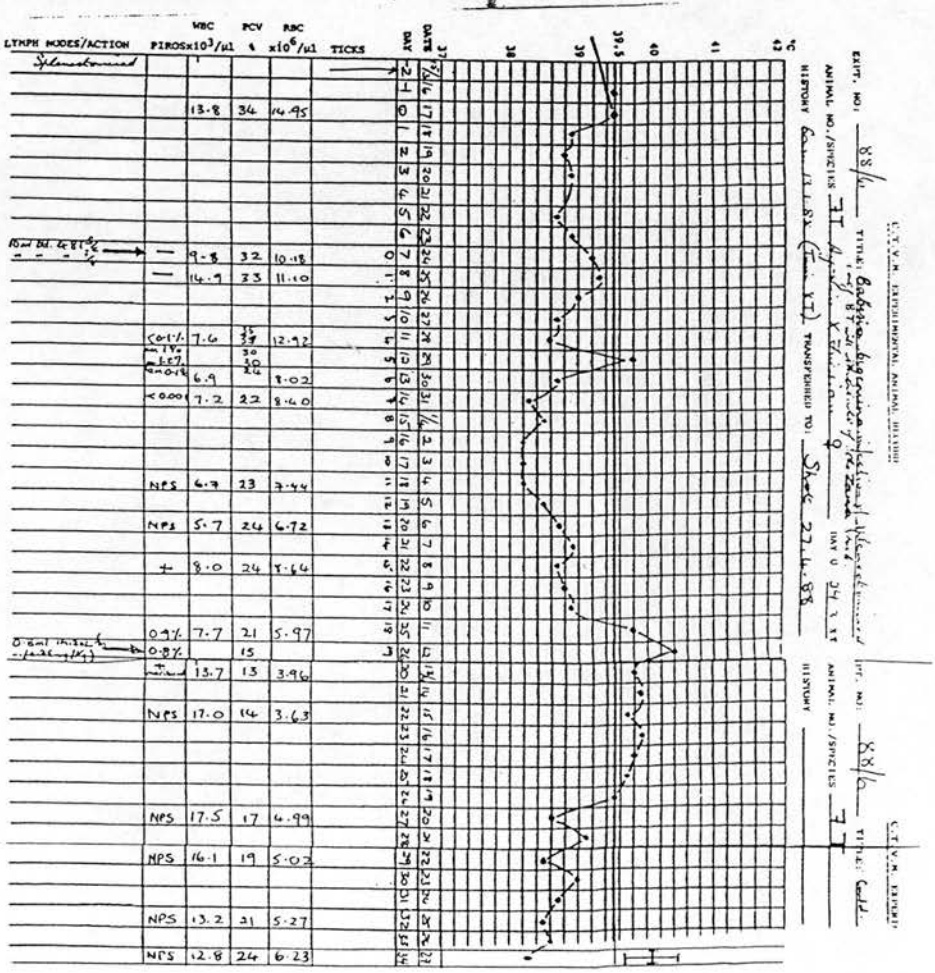
ANIMAL NO./SPECIES U44 (4mo. Friesian) DAY 0 22.9.86

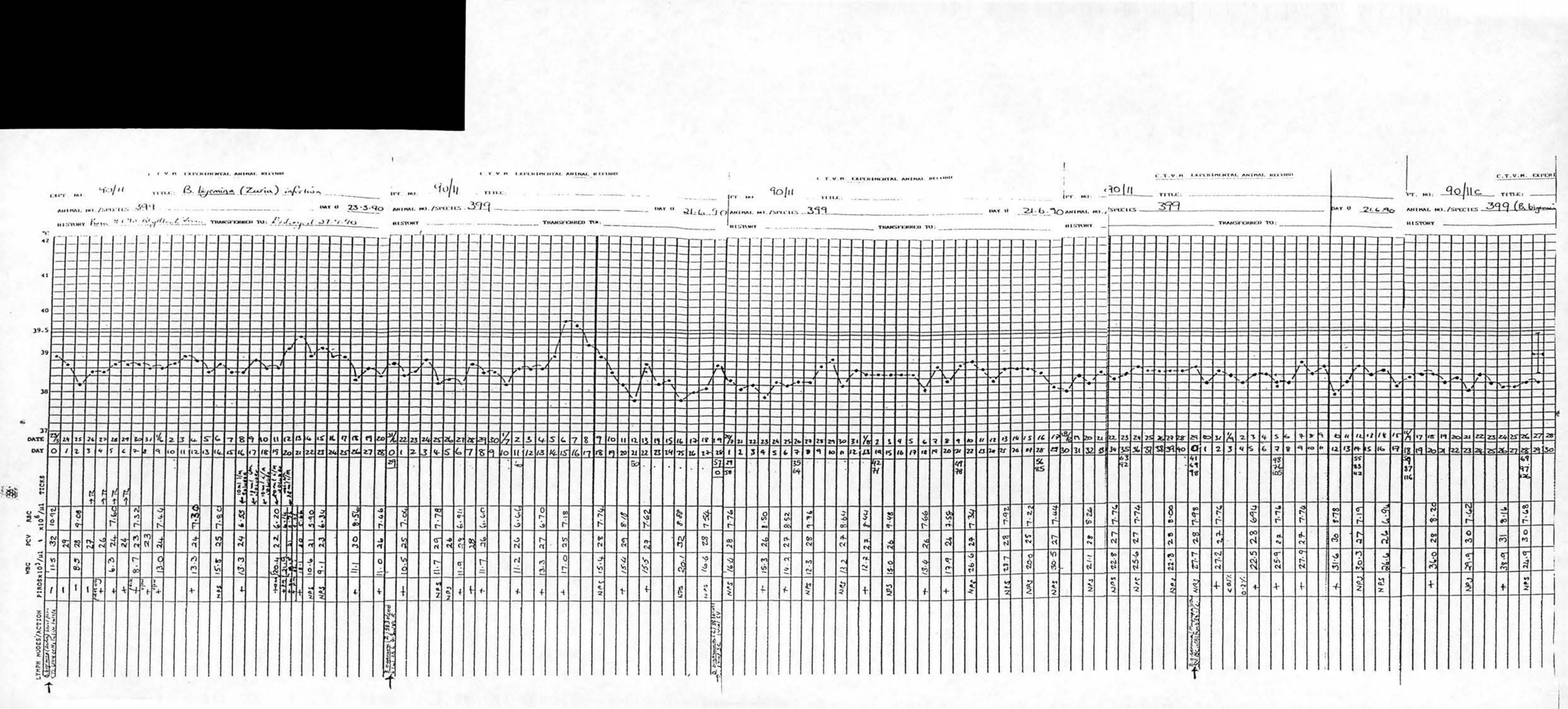
HISTORY _____ TRANSFERRED TO: _____

EXPT. NO: 86/ ANIMAL NO./SPE HISTORY


°C
42
41
40
39.5
39
38
37
DATE
DAY
WBC
PCV
RBC
TICKS
LYMPH NODES/ACTION
P:NOS x10³/µl
x10⁶/µl
Splenectomy
1-4 Trypanosomes Blood IV (Dan '77)
2-4 Babesia bigemina blood Ke. 1984
NPS
NPS
NPS


LYMPH NODES/ACTION
WBC PIROSx10³/µl
PCV %
RBC x10⁶/µl
TICKS
DAY
TEMP °C
EXPT. NO:
TITLE:
ANIMAL NO./SPECIES
HISTORY
TRANSFERRED TO:
TRANSFER
C.T.V.M. EXPERIMENTAL ANIMAL HOUSING


LYMPH NODES/ACTION
WBC PIROSx10³/µl
PCV %
RBC x10⁶/µl
TICKS
DAY
TEMP °C
EXPT. NO:
TITLE:
ANIMAL NO./SPECIES
HISTORY
TRANSFERRED TO:
C.T.V.M. EXPERIMENTAL ANIMAL HOUSING


C.T.V.R. EXPERIMENTAL ANIMAL RECORD
EXPT. NO. 90/11
TITLE: B. bigemina (Zuria) infection
ANIMAL NO./SPECIES: 399
DAY 0 23.3.90
HISTORY: Bom. 8 CVO Highfield Farm
TRANSFERRED TO: Edinburgh 27.4.90
C.T.V.R. EXPERIMENTAL ANIMAL RECORD
EXPT. NO. 90/11
ANIMAL NO./SPECIES: 399
DAY 0 21.6.90
HISTORY
TRANSFERRED TO:
C.T.V.R. EXPERIMENTAL ANIMAL RECORD
EXPT. NO. 90/11
ANIMAL NO./SPECIES: 399
DAY 0 21.6.90
HISTORY
TRANSFERRED TO:
C.T.V.R. EXPERIMENTAL ANIMAL RECORD
EXPT. NO. 90/11
ANIMAL NO./SPECIES: 399
DAY 0 21.6.90
HISTORY
TRANSFERRED TO:
C.T.V.R. EXPERIMENTAL ANIMAL RECORD
EXPT. NO. 90/11c
ANIMAL NO./SPECIES: 399 (B. bigemina
HISTORY
°C
42
41
40
39.5
39
38
37
DATE
DAY
LYMPH NODES/ACTION
PIROS
WBC x10³/µl
PCV
RBC x10⁶/µl
PICKS
MBC

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/11a TITLE: B. bigemina (Zaria) infection

ANIMAL NO./SPECIES 583 Ayrshire ♂ DAY 0 14.6.90 ANIMAL NO./SPECIES 583

HISTORY Splenectomised 28/5/90 TRANSFERRED TO: Pirbright 27.9.90 HISTORY TRANSFERRED TO:

°C 37 38 39 40 41 42

Day	PIROPLASMS	WBC ×10³/µl	PCV %	RBC ×10⁶/µl	TICKS
0	—	13.2	35	12.24	
1	—	10.7	36	12.56	
2	+(hairy)				
3	+		32		
4	<0.1%	11.1	33	11.50	
5	0.6%		32		
6	1.0%	12.4	25	8.58	
7	0.4%	7.2	19	6.19	
8	0.2%	9.1	10	4.15	
9	0.2%		11		
10	NPS	10.7	16	3.87	
11	NPS		16		
12	NPS	9.2	17	3.56	
13	± 2 dyed cells/fld		19		
14	NPS	10.5	20	3.89	
15					
16					
17	NPS	11.0	26	5.20	
18					
19	NPS	10.7	27	5.22	
20					
21	NPS	10.3	29	7.00	
22					
23					
24	NPS	11.2	29	7.66	
25					
26	NPS	9.9	31	7.66	
27					
28	NPS	9.6	32	8.12	
29					
30					
31	NPS	12.2	35	9.08	
32					
33	NPS	10.8	34	8.96	
34					
35					
36	NPS	11.8	33	8.70	
37					
38					
39	NPS	14.7	33	9.52	
40	NPS	12.2	35	9.76	
41					
42	NPS	12.3	35	10.50	
43					
44					
45	NPS	10.0	35	11.26	
46					
47	NPS	9.0	33	9.06	
48					
49					
50	NPS	10.3	31	14.48	
51					
52					
53	NPS	10.0	34	9.50	
54					
55	NPS	11.1	34	9.81	
56					
57	NPS	11.3	34	9.32	
58					
59					
60	NPS	11.1	34	9.72	
61					
62	NPS	10.7	32	9.31	
63					
64	NPS	12.7	33	9.38	
65					
66					
67	NPS	12.9	32	9.92	
68					
69	NPS	13.0	32	9.42	
70					
71					
72	NPS	12.3	32	9.60	
73					
74					
75	NPS	10.4	34	9.66	
76					
77					
78	NPS	12.1	32	9.50	
79					
80	<0.1%	7.4	32	9.40	
81					
82	NM				
83	NPS	12.8	34	8.54	
84					
85	+	11.1	30	9.40	
86					
87	0.2%	9.8	31	9.28	
88					
89	0.1%				
90	<0.1%	12.3	22	6.72	
91					
92	+	12.2	19	5.53	
93					
94	+	9.5	19	5.53	
95					
96					
97					
98					
99	0.2%	9.9	19	4.74	
100	<0.1%		16-18		
101	+		17		
102	+	25.1	18	3.69	
103	+				
104	+				
105	+	12.6	21	6.06	
106					
107	0.1%	9.6	21	3.99	
108					

B. bigemina (Zaria) bloodworm 1ml SC, 1ml IV.

B. bigemina (Muguga) 10ml SC, 1ml IV, PBS 1ml IV

Blood sp. for B. bigemina (Zaria) + buffy coat cryopreserved

20ml 1/5 squash
25ml 1/10 squash

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/11a TITLE: B. bigemina (Zaria) infection (contd)

ANIMAL NO./SPECIES 583 DAY 0 14.6.90 ANIMAL NO./SPECIES 583

HISTORY TRANSFERRED TO: HISTORY TRANSFERRED TO:

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/11a TITLE: B. bigemina (contd)

ANIMAL NO./SPECIES 583 DAY 0 14.6.90

HISTORY TRANSFERRED TO:

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/11a TITLE:

ANIMAL NO./SPECIES 583

HISTORY TRANSFERRED TO:

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/9 TITLE: Production of immune responses to B. bovis and B. bigemina

ANIMAL NO./SPECIES 397 Ayrshire ♂ DAY 0 20.4.90

HISTORY from 11.12.89 BGyll herd TRANSFERRED TO:

B. bigemina (Zana)

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/9 cont. TITLE:

ANIMAL NO./SPECIES 397 DAY 0 20.4.90

HISTORY TRANSFERRED TO:

C.T.V.M. EXPERIMENTAL ANIMAL RECORD

EXPT. NO: 90/9 cont'd. TITLE:

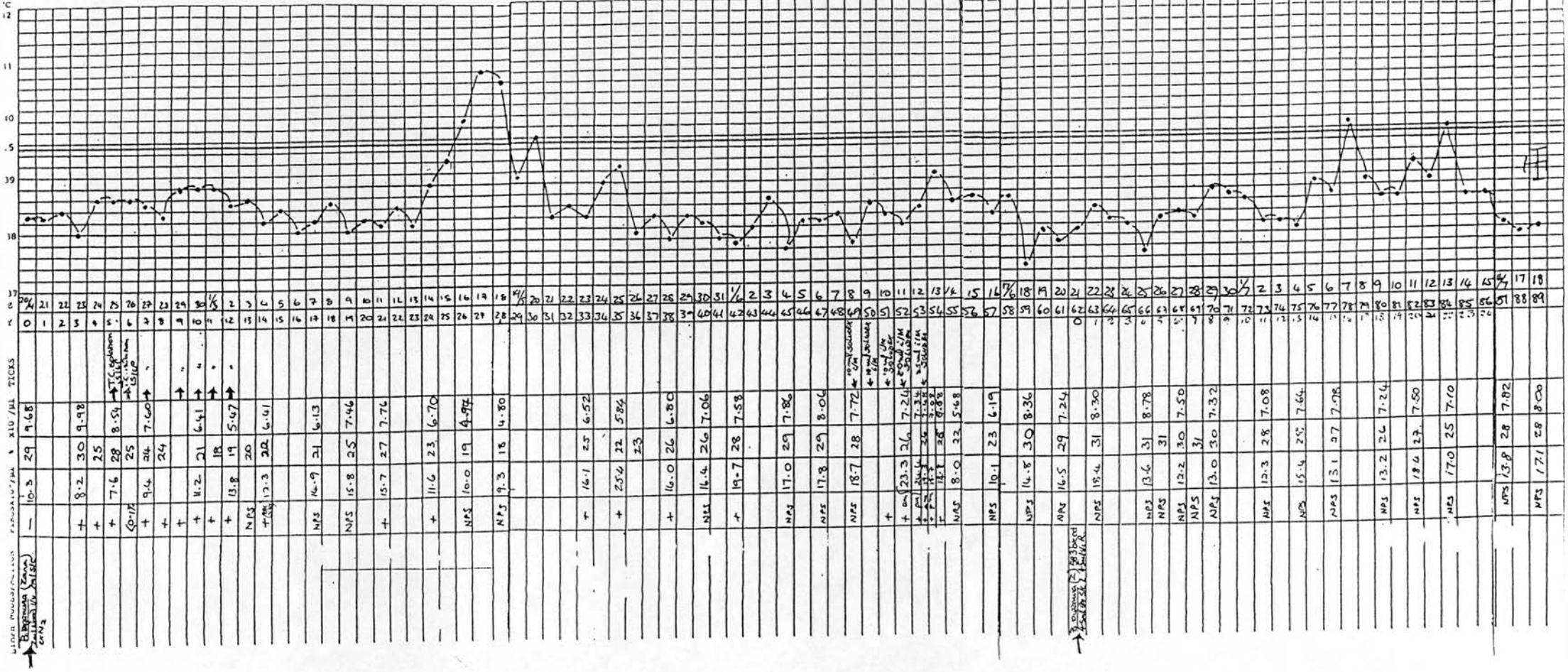
ANIMAL NO./SPECIES 397 DAY 0 20.4.90

HISTORY TRANSFERRED TO:

EXPT. NO: 9

ANIMAL NO./

HISTORY


°C
42
41
40.5
39
38
37
% RBC/µl x 10⁶
TICKS
B.bigemina (Zana) 2x10⁸ i.v.
B.bovis (Entebbe) 2x10⁸ i.v.
T.C. injection
T.C. injection
T.C. injection